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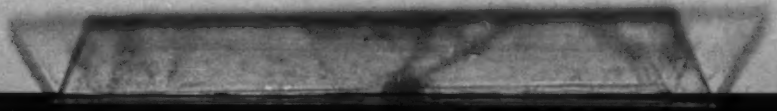

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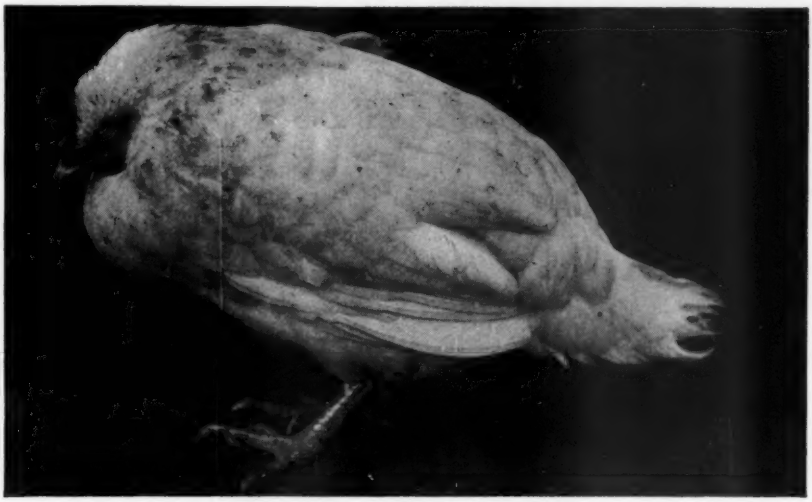

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VOL. XIII APRIL, 1952 No. 47

A Complement-Fixation Test for Leptospirosis in Cattle

CHARLES J. YORK, D.V.M., Ph.D.

Ithaca, New York

LEPTOSPIROSIS, RECOGNIZED during recent years as a specific disease of cattle in Russia,[1] Palestine,[2] and Australia,[3] was first observed in the United States in 1944 in Connecticut.[4] Since then, other outbreaks of this disease have been seen in widely separated areas of the United States,[4-9] and successful cultivation of strains of Leptospira from one of these outbreaks was reported in 1948.[7] Later work with this strain proved that it was different antigenically from the *Leptospira bovis* found in Palestine.[10] Recent work indicates that it seems more closely related to *Leptospira pomona*, reported in Australia as the cause of disease in cattle and man, and found also in swine and man in some parts of Europe.[11]

Actual incidence of this disease in the cattle population of any area has not been determined as yet. One major difficulty in this determination has been lack of a suitable serological test. The standard agglutination-lysis test described in various bacteriology textbooks was not considered satisfactory, both because of certain technical difficulties and the possibility of questionable interpretation. The need for an objective yet reliable serological test for bovine leptospirosis became necessary. Complement-fixation tests for human leptospirosis[12-18] used a variety of antigens prepared by several methods. Although these antigens for human leptospirosis might be used for work with cattle, an antigen making use of Leptospira organisms cultivated in eggs was employed. It is the purpose of this paper, therefore, to describe this complement-fixation test for cattle.

From the Veterinary Virus Research Institute, New York State Veterinary College, Cornell University, Ithaca.

THE COMPLEMENT-FIXATION TEST

Preparation of Constituents.—In order to standardize the various constituents in the test, complement was titrated with excessive amounts of amboceptor and in the presence of antigen. Next, amboceptor and then antigen were measured.

Sheep cells were collected in Alsever's solution from the same sheep that was used as the source of amboceptor. These cells were washed three times and stored in a 50 per cent suspension in Alsever's solution at 4 C. Cell suspensions thus prepared usually kept in good condition for at least six weeks. When used in tests, a 2 per cent suspension was prepared and 0.2 cc. added to each tube in the test.

To prepare complement, 30 or more male guinea pigs were bled by cardiac puncture; serum was removed four hours after collection, pooled, and then stored in sealed ampules under dry ice refrigeration for later use. A sample was removed, thawed, and a determination made of its unit value. The complement was diluted 1 : 20 and titrated in duplicate, with .06 cc. in the first tube and an increment in each succeeding one of .02 cc. Then, a 1 : 5 dilution of antigen and saline was added to make a final amount of 0.6 cc. in each tube. The mixture was incubated at 37 C. for one-half hour and then 0.4 cc. of sensitized cells, which consisted of equal portions of 2 per cent sheep cells and an excess of amboceptor, were added. One unit of complement was taken as the smallest amount which would cause complete hemolysis of the sheep cells. Two units contained in 0.2 cc. were always used in each tube in the test.

Red cells from a single sheep were used to make amboceptor, which was prepared in rabbits. A series of five inoculations of 10 per cent washed cells were given each rabbit every five days. The rabbits were bled by cardiac puncture five days after the last injection, serum obtained, mixed with an equal part

of glycerin, and kept under refrigeration until used. Serial twofold dilutions of amboceptor, which started with a 1 : 1,000 dilution, were prepared and 0.2 cc. were added to each tube that contained 0.6 cc. of a mixture of previously incubated complement (2 units), 0.2 cc. of a 1 : 5 dilution of antigen, and 0.2 cc. of saline. A 2 per cent suspension of sheep cells was then added and the mixture incubated again for one-half hour. The highest dilution of amboceptor which caused complete hemolysis of the sheep cells was considered one unit. Two units as contained in 0.2 cc. were always used for each tube in the test.

In preparing antigen, hens' eggs that had been embryonated for eight days were each inoculated with 0.5 cc. of fluid from eggs infected with Leptospira organisms (strain T262) that had been maintained continuously in eggs since 1947. After a further incubation for five days, chorioallantoic fluid was obtained without chilling the embryos and centrifuged at 2,000 revolutions per minute (r.p.m.) for ten minutes to remove blood cells and tissue fragments. The supernatant was decanted, formalin added to a concentration of 0.3 per cent, and again it was centrifuged at 10,000 r.p.m. for one hour. The sediment, which consisted primarily of leptospiral organisms, was resuspended to one tenth of the original volume in saline that contained 0.1 per cent formalin. This constituted the antigen for the test. In titrating the antigen, two series of tubes were prepared which consisted of 0.2-cc. amounts of antigen in dilutions of 1 : 2, 1 : 4, 1 : 8, 1 : 10, and 1 : 12. To each tube in one series was added 0.2 cc. of a 1 : 8 dilution of known positive leptospiral antiserum, and to the other series, the same dilution of known negative serum was added in like manner for control. Complement was added, mixed thoroughly, and incubated in a water bath at 37 C. for one-half hour. Sensitized cells were added, incubated again for one-half hour, and then examined. The highest dilution of antigen that showed complete fixation of complement was considered one unit. The antigen should show no fixation with negative serum, and this never occurred. In the actual test, two units of antigen contained in 0.2 cc. were always used for each tube in the test.

Only cattle serums were tested. After removal from the blood clot with a minimum of lysis, each sample was inactivated at 56 C. for twenty minutes and then stored under refrigeration—either at 4 C., if tested immediately, or frozen, if held longer than two weeks. In the test, serial twofold dilutions were prepared and 0.2 cc. added to each tube in the test.

Thus, in an actual test, 0.2 cc. of each of the constituents, standardized as discussed previously, and 0.2 cc. of serum to be tested were placed in a tube to make a total of 1 cc. Appropriate controls, especially for those tubes which contained two units, one unit, and one-half unit of complement and the serum to be tested, were included in each test and were deemed essential.

Evaluation of the Test.—In order to evaluate the usefulness of this test, serums were obtained from 12 calves before inoculation and again three to four weeks afterwards. In addition, serums were procured from 214 animals in six herds that showed clinical evidence of leptospirosis. Of these, 32 samples were taken during the acute phase and two to three weeks later during convalescence. As a further proof that leptospirosis existed in some of these animals, actual isolation of Leptospira was made from 6 animals in the different herds. Serums were also obtained from a herd of 21 cattle maintained free of infection at this laboratory. These results are shown in table 1.

As can be seen in table 1, all serums taken from calves before inoculation failed to fix complement, whereas, after inoculation, all fixed with dilution end points that ranged from 1 : 8 to 1 : 128. There was no fixation by serums from any cow in the disease-free herd. Serums taken from cattle during or shortly after clinical illness failed

TABLE 1—Result of Complement-Fixation Tests with Serums of Normal, Experimentally Inoculated, and Naturally Infected Cattle

Cattle from which serums obtained		No. of samples	Dilution of serums fixing complement						
			1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256
Experimental	Pre-inoculation	12	0/12*	0/12	0/12	0/12	0/12	0/12	0/12
	Postinoculation	12	12/12	11/12	10/12	10/12	8/12	8/12	0/12
Disease-free herd		21	0/21	0/21	0/21	0/21	0/21	0/21	0/21
Infected herds	Acute phase	32	8/32	4/32	2/32	0/32	0/32	0/32	0/32
	Convalescent phase	214	214/214	209/214	194/214	183/214	160/214	28/214	18/214

*Numerator indicates the number of samples showing complete fixation of complement at a given dilution. The denominator indicates the number of samples tested.

to fix complement or fixed in very low dilutions. After recovery from natural infection, complement was fixed by dilutions that ranged from 1 : 8 to 1 : 256.

Specificity of Test.—Serums were obtained from cattle known to have recovered from virus diarrhea and from other cattle that had had winter dysentery. Serum samples known to agglutinate Brucella organisms and vibrionic abortion antigens also were obtained and tested. Serums that fixed complement with the leptospiral antigen were included as controls. These results are summarized in table 2.

TABLE 2—Complement-Fixation Tests with Leptospiral Antigen, Using Serums of Cattle Convalescent from Other Diseases

Disease	No. cattle tested	Complement-fixation results
Brucellosis	20	Negative
Virus diarrhea	12	Negative
Winter dysentery	15	Negative
Vibrionic abortion	10	Negative
Leptospirosis	12	1 : 8 to 1 : 128

It can be seen that serums from cattle convalescent from either winter dysentery or virus diarrhea failed to fix complement. Serums that agglutinated Brucella and Vibrio antigens also failed to fix complement. Leptospiral antiserums fixed as usual. It appears, therefore, that the complement-fixation test for leptospirosis is specific.

Comparison with Agglutination-Lysis Test.—Serums from 35 cattle that fixed complement were tested by the agglutination-lysis test. In addition, 20 serums that failed to fix complement were also tested. In the agglutination-lysis test, a suspension of living Leptospira was mixed with various dilutions of serum, incubated for one hour, and then examined by dark field microscopy. The last dilution of serum in which more than 50 per cent of the organisms were lysed or agglutinated was selected as an end point. The results of these tests are presented in table 3.

As can be seen in table 3, there is a close correlation between the results of the complement-fixation test and the agglutination-lysis test, with serums having high or low end point-dilution values in fixing complement being matched with corresponding high or low agglutination-lysis end point dilutions.

DISCUSSION

Details in the preparation of materials and methods of conducting a simple complement-fixation test for bovine leptospirosis have been described. Results obtained thus far indicate that the test is specific and accurate and, in routine diagnosis and in surveys for incidence, it has proved valuable.[19]

TABLE 3—Comparison of Agglutination-Lysis Test with Complement-Fixation Test

No. serums tested	End point dilutions	
	Complement-fixation	Agglutination-lysis
20	0	0 to 1 : 80
4	1 : 4	1 : 200
5	1 : 8	1 : 200 to 1 : 400
3	1 : 16	1 : 800
10	1 : 32	1 : 800 to 1 : 1,600
4	1 : 64	1 : 1,600 to 1 : 3,200
6	1 : 128	1 : 1,600 to 1 : 6,400
3	1 : 256	1 : 6,400

Antigenic comparisons have not been made of Leptospira organisms cultivated in eggs with those maintained in culture. The antigen used herein made use of Leptospira cultivated in eggs, since eggs are readily available and would appear to be more standard than culture mediums that necessarily would require serum from a variety of sources. Furthermore, pathogenicity is soon lost by cultivation in any cell-free medium but is retained by cultivation in eggs, and this may have some bearing on antigenicity.

A comparison was made between the complement-fixation test and the agglutination-lysis test, with a close correlation between dilution end points for the two tests. However, the complement-fixation test has several advantages over the agglutination-lysis test: (1) it is an objective test; (2) there is a sharp and distinct difference between positive and negative serums, thus avoiding a large portion of the weak agglutination-lysis reactions that are now regarded as suspicious; and (3) it should be a more useful method for small laboratories, especially if antigen becomes available commercially, since then it will not be necessary to maintain living cultures of Leptospira organisms.

SUMMARY

A complement-fixation test for the serological diagnosis of bovine leptospirosis is described. Since serums from uninoculated

and noninfected cattle failed to fix complement, while serums from animals that had recovered from experimental and natural infection did, the test is considered reliable. Specificity is indicated by lack of cross reactions with serums from cattle that had recovered from brucellosis, vibriosis, virus diarrhea, and winter dysentery. Accuracy compares favorably with the agglutination-lysis test but, since the complement-fixation test is objective and more easily performed, it should prove useful in diagnosis.

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A Severe Mastitis Problem Associated with Cryptococcus Neoformans in a Large Dairy Herd

W. D. POUNDEN, D.V.M., Ph.D.; J. M. AMBERSON, E.M., M.D.; R. F. JAEGER, A.B.

SOME OF THE yeast and yeastlike fungi have been associated with mastitis. Rolle[9] probably was the first to draw attention to such cases.

A number of fairly mild cases in which a species of Trichosporon was incriminated were described by Murphy and Drake.[7] Anderson and Jorgensen[1] concluded that a problem involving a yeast infection (Torulaceae) of the udders of several cows resulted from the use of infected water as a solute for penicillin that was infused during treatment for mastitis. Another mastitic-herd problem encountered by Stuart[10] involved an organism of the genus Candida. The attacks followed treatment with penicillin infusions, but the yeast was thought to have originated from brewer's grains being fed the cows. Burkey *et al.*[2] refer to the occurrence of limited numbers of cases of mastitis caused by yeast and yeastlike organisms in a large herd under observation for several years. Such cases have been encountered by us on a few occasions. The symptoms were not as severe as those described by Stuart[10] but were more so than those observed by Murphy and Drake.[7] With or without treatment, the clinical symptoms persisted for approximately a week, a period similar to that mentioned by Stuart.[10]

The present report concerns a problem in a dairy herd composed of approximately 235 head of Holstein-Friesian cattle in which the diseased udders were infected with an organism coming under the same general category. The attacks were variable in severity and, in many instances, were far more acute and persistent than those described by others mentioned previously.

The organism was determined by Emmons[3] to be *Cryptococcus neoformans*. This is the causative agent of human cryptococcosis, the lesions of which frequently involve the central nervous system and the lungs.[3, 8] The organism is encountered occasionally as a pathogen in domestic animals, as mentioned by Emmons,[3] but no reports were found in which it was associated with mastitis.

The herd was housed in five barns and was milked by machine. Almost all the animals were in good condition and were receiving rations composed of second quality alfalfa hay free choice, corn silage at the rate of 30 lb. per day, along with 3 lb. of beet pulp, molasses, and grain. The grain was approximately 20 per cent protein, contained corn, bran, barley, oats, and protein concentrates, and was fed at the rate of approximately 1 lb. of grain per 3 lb. of milk produced per day, 18 lb. per day being the upper limit fed.

The practice that had been followed for handling mastitis cases was to infuse, in routine manner, all visibly affected quarters with aqueous solutions of penicillin. The operators had been confronted, during a period of approximately two months prior to seeking assistance, by increasing numbers of severe cases which differed from those usually encountered in their herd and which failed to respond to this treatment.

A preliminary bacteriological examination, in which blood agar and Sabouraud's medium of quarter milk samples from 10 of the affected animals were used, showed that considerable numbers of spherical yeast cells were present in 23 samples from 9 of the animals and that nine other samples were infected with streptococci, staphylococci, or coliform organisms. The presence of the yeast cells was demonstrated in samples from 28 head when a check was made of all milking animals. Samples were not available from a few cows which were thought to have had similar attacks, as they had been disposed of, and a few others had become dry since the attacks had occurred.

EXTENT OF PROBLEM

Forty-eight animals had abnormal conditions of their udders which were associated with the yeast infection during the first four months (November, 1950 to March, 1951) that the herd was under our observation. In 5 of them, the condition never exceeded in severity the temporary production of some visibly abnormal

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milk. In the majority, the affected quarters became severely swollen. Four other cows were observed to shed the organisms, but visible changes in the udders or milk were not noted. An additional 25 cows had temporary abnormal conditions of their udders or milk during the same period. Various bacteria, including streptococci, staphylococci, and coliform organisms were isolated from samples from 8 of these. The remainder had not been examined bacteriologically at the time of the abnormality. Cryptococcus organisms were present in samples collected from 4 of these latter cows when checked a few weeks later.

During the following two months (March to May), 7 more cows had abnormal udder conditions of limited severity due to infection with the Cryptococcus. One or more quarters of 44 others were observed to shed the organisms in the absence of visible abnormalities. Cryptococcus organisms were detected for the first time in milk samples from but 3 other animals during the next three months. Clinical symptoms were absent in all 3. This made a total of 55 cows which had visibly abnormal udders or udder secretion resulting from the cryptococcal infection and 51 others which were found to shed the infection in their milk on at least one occasion, thus making a total of 106 animals that have been involved so far.

Udder samples from previously affected animals were found to be free of the cryptococcal infection in increasing numbers, beginning with examinations in March. By the end of September, the herd contained 12 animals which had become infected and had shown visible symptoms of the disease yet had recuperated to the extent that Cryptococcus organisms could not be recovered from quarter milk samples obtained on two or more monthly examinations. In 1 of these animals, a severe attack in one quarter had rendered this gland useless, at least for the remainder of the lactation. From an additional 7, formerly visibly affected animals, samples free of the Cryptococcus were obtained on a single examination. Such samples from 17 cows which previously had been found to shed the organisms without clinical symptoms were also obtained at least once.

Studies carried out on more than 60 cultures obtained during the early months of the outbreak had shown the existence of minor variations in the physiological characteristics between groups of the organisms. However, all produced lesions in mice which were typical of *C. neoformans*.[5] Further studies conducted during later months, when clinical cases were both less numerous and less severe, indicated that, in the meantime, changes had not occurred in the characteristics of the organisms.

In January and February, 11 cows had to be culled because of serious damage to their udders caused by the cryptococcal infection. Seven others had followed by the end of August, making a total of 18 for the first eight months of 1951. Several additional animals were removed for apparently similar reasons during the earlier months of the outbreak when bacteriological records were not always available.

CLINICAL SYMPTOMS

The clinical symptoms that followed infection of the udder with Cryptococcus were variable, as is true of most mastitis infections, and in mild attacks they frequently resembled in many respects those accompanying other types of infections. In severe attacks, which were numerous during the first few months of the outbreak, the symptoms differed appreciably from those observed in more usual cases. These attacks were characterized by severe swelling and firmness of the gland that took several days to reach maximum intensity and then persisted for extended periods. Involvement of more than one quarter was common and, in some instances, all four were seriously affected.

Frequently, the first indication that an abnormal condition was developing was swelling and increased firmness of secretory tissues in the dorsal parts of the gland. The swollen condition progressively involved the remainder of the quarter during the next one to two weeks and was followed by the more intense stage, which frequently persisted for a month or more. During this phase, the affected quarters were extremely swollen and distended. The subcutaneous tissues of the udder and, sometimes also, those anterior to the udder generally became edematous. Milk production gradually decreased, including that from unaffected quarters when the attacks were severe. Animals producing 60 lb. per day at the onset of the symptoms sometimes yielded less than a third of this within a month and went completely dry by the end of another month.

Readily visible changes in the milk generally were not detected until one to two weeks after the swelling had been noted. In some instances, the first noticeable sign of milk change was the presence of small flakes that were visible on the walls of sample bottles giving an appearance rather similar to that shown in a photograph by Murphy and Drake.[7] Watery serum accompanied by flakes was all that could be

obtained from some severely affected quarters. Some samples which at first appeared fairly normal would on standing separate into watery liquid and a mass of gray-white substance which floated between the cream layer and the watery liquid (fig. 1, center vial). The secretion that was characteristic of severe attacks after they had persisted for three weeks or more and

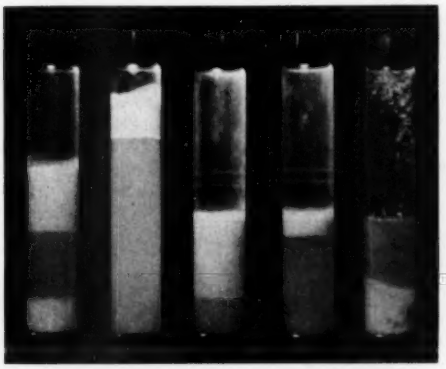

Fig. 1—Udder cryptococcosis. Abnormal udder secretions.

when milk production had been seriously curtailed was gray-white, highly viscid, and mucoid. Figure 2 shows samples of this material and demonstrates its high viscosity through its slowness to move down through the inverted tube. When this material was present, difficulty was experienced in injecting any quantity of liquid into the glands in excess of a few cubic milliliters.

The animals suffered considerable discomfort in severe attacks. Some were forced to stand with their hindlegs abnormally spread apart because of the extreme distention of their udders (fig. 3). Temperatures generally did not deviate more than 2 to 3 degrees above normal, even during the more severe stages. Depression, inappetence, and weakness were seldom present to any marked degree. They appeared to occur only when the animals were suffering intense local discomfort caused by the excessive swelling of the udders and not because of the presence of toxic substances, such as may exist in severe attacks caused by coliform organisms or in gangrenous cases associated with staphylococci.

Palpation of the supramammary lymph nodes showed they frequently were enlarged. Examinations at autopsy[4] showed the marked extent to which this had oc-

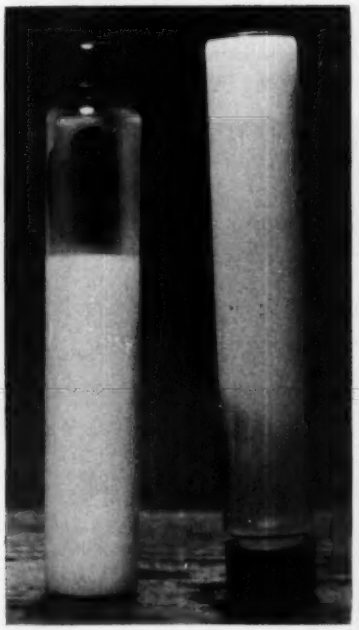

Fig. 2—Udder cryptococcosis. Gray-white, viscid abnormal udder secretion.

curred in some instances (fig. 4). This condition of the lymph nodes persisted for many subsequent weeks.

INFECTION ORIGIN AND SPREAD

An effort made to locate the origin of the infection was unsuccessful. It was not possible to determine with any certainty the barn or cow in which the infection started. Its spread was erratic and did not follow closely any definite pattern, such as the stanchion order or the order of milking. The possibility was considered that treatments with penicillin of attacks of mastitis caused by other organisms might be, in some manner, associated with the cryptococcal infections, inasmuch as some connection between yeast infections and such treatments has been mentioned on two occasions.[1, 10] There did not appear to be an essential association, as many of the

infections had not been preceded by penicillin treatments.

A search failed to reveal any Cryptococcus organisms in samples of feed, feces, and rumen contents[3], or from various possible sources in the environment of the cows. Furthermore, physical examinations of the personnel working with the herd failed to demonstrate a human source of infection.

Fig. 3—Udder cryptococcosis. Severe distention of udder causing legs to be spread apart.

Thus, the only detected source of infective material for new cases was the udders of infected animals. There were no apparent reasons for believing the organisms gained entrance to the udders other than through the teat orifice.

The conditions usually considered to be the cause of the transmission of mastitis organisms between barns and cows were present. However, the groups of cows housed in each barn were kept separate and movement of animals between barns was somewhat restricted. Although milkers were assigned to a single barn, a possibility for transfer of the infection by human means existed, as one man acted as relief person by rotating weekly among all five barns, and all mastitis treatments were administered by the herdsman and his assistant.

The disinfectants used for washing udders and for rinsing the milking machine teat cups between cows were inadequate for controlling the Cryptococcus, as cultural studies demonstrated the existence of the organisms in the buckets and teat cups. Sterilization of equipment used in making penicillin udder infusions was not adequate, although culturing failed to demonstrate the presence of the Cryptococcus on or in syringes, needles, or in the water in which the penicillin was dissolved on the one occasion a check was made.

Fig. 4—Udder cryptococcosis. Enlarged mammary lymph nodes containing granulomatous lesions. (The accompanying ruler is 6 in. long.)

With regard to the readiness of growth of the yeast in raw milk, which might be a related factor in the invasion of the gland or in the development of the abnormal udder condition, it was observed that samples of milk from 4 uninfected cows, 2 from a different herd, were comparatively unsuitable as mediums until 5 per cent or more of blood serum was added to them. This might indicate that teat and udder injuries could play a part in the susceptibility of the udders to the infection. At the time of the first inspection of the herd, a number of teats had minor cracks and erosions at the orifice. An additional observation, in regard to the influence of

teat injuries and also concerning the speed with which the infection could gain access to an udder, was made on a cow purchased while in milk. Seven days after arrival in the herd, she bruised and lacerated the end of one of her teats. The treatment she received included infusions of penicillin. A milk sample collected 14 days later contained *C. neoformans*.

CONTROL PROGRAM

The control program consisted of repeated cultures of samples from all animals to detect new infections, segregation of infected from healthy stock, removal from the herd of animals affected to the extent that their retention was unprofitable, use of sanitary milking procedures, and correct machine operation. Other details given consideration included increased emphasis on the use of only sterilized equipment for mastitis treatments and greater care in preventing the infection from being carried by the personnel from cow to cow or barn to barn. Additional help was sought in treatments. As precautionary measures, an effort was made to supply hay of better quality and to reduce the quantity of grain as well as the protein content of the grain. Freshly cut green feed, mostly alfalfa, was provided daily beginning approximately on the first of May.

TREATMENTS INVESTIGATED

A number of antibiotics and drugs were examined in a search for inhibitors of growth of *C. neoformans* which would be suitable also for treatment. In the first trials, various materials were added to 20-ml. samples of aseptically collected raw milk along with 1 ml. of bovine serum and 1 ml. of broth culture of the Cryptococcus. Following twenty-four hours' incubation, the samples were streaked on blood agar and incubated for three days. The number of colonies which appeared on the blood agar plates served as an indicator of the relative growth in the milk containing the various agents.

A summary of these trials is presented in table 1. Numerous colonies of the Cryptococcus developed in plates from milk samples containing penicillin, streptomycin, both of these together, aureomycin, and bacitracin. Relatively few colonies developed in those from samples containing either bi- or tri-phenyl-tetrazolium, antibiotic 272,[a] and sopronol.[b] In the tubes containing ethylene oxide, no growth resulted.

Another antibiotic, actidione,[c]® was added to blood and Sabouraud's mediums in concentrations as high as 10 mg. per 15 ml. of agar. The Cryptococcus grew readily in all dilutions, which was not as expected from the findings of others in regard to *C. neoformans*.[5] However a check made later using the paper disc method[6] demonstrated inhibitory action.

All the products, except ethylene oxide, having inhibitory action in milk were sufficiently mild in their action on udder tissues to justify clinical trial. When infused into the glands of a limited number of severe cases, none proved to be of real help in alleviating the clinical symptoms or in markedly reducing the numbers of the organisms being shed in the udder secretions. A slight beneficial effect seemed to be obtained from four infusions of antibiotic 272 given two per day of 50,000 units each, from three infusions of 30 ml. each of a 1 : 100 solution of bi-phenyl-tetrazolium given at consecutive milkings, and from three infusions of 30 ml. each of sopronol given the same way.

A 1-Gm. dose of an antihistiminic, pyranisamine maleate,[d] was given intravenously to an acute case. This was repeated next day, but in neither instance was any effect apparent. Cortisone[e] was

[a] Sharpe & Dohme Company.
[b] Wyeth Company.
[c] Upjohn Company.
[d] Merck & Company.
[e] Cortone,® Merck & Co., Inc.

TABLE 1—Sensitivity of Cryptococcus Neoformans from Mastitis Cases as Determined by Growth in Raw Milk Containing 5.0 Per Cent Blood Serum

Agents	Conc./ml. milk	Colonies on blood agar plates
Penicillin	250 units	Many
Streptomycin	2.5 mg.	Many
Penicillin and streptomycin	250 units and 2.5 mg.	Many
Aureomycin	1.25 mg. and 0.125 mg.	Many
Bacitracin	200 units and 20 units	Many
Bi-phenyl-tetrazolium	1 : 10,000	Few
Tri-phenyl-tetrazolium	1 : 10,000	Few
Antibiotic 272	10 units	Few
Sopronol	0.05 ml.	Few
Ethylene oxide	0.025 ml.	None

also administered intramuscularly in a 500-mg. dose to the same animal and to another one which was seriously affected. This was repeated next day in the second animal, but in both instances without noticeable effect.

Further *in vitro* studies of possible inhibitory agents were made using the paper-disc plate method,[6] in which 0.08 ml. of the solution under test is placed on each paper disc. Some of the antibiotics were included in both trials. A summary is presented in table 2.

Three substances, mapharsen,[f] bismarsen,[g]® and merthiolate were observed to exert inhibitory powers and were given limited clinical trial.

TABLE 2—Sensitivity of Cryptococcus Neoformans from Mastitis Cases to Various Agents as Determined by Paper-Disc on Blood Agar

Agent	Conc./ml. on paper-disc	Relative inhibition
Penicillin	0.5 to 10.0 units	0
Streptomycin	0.5 to 10.0 mg.	0
Di-hydrostreptomycin	0.5 to 10.0 mg.	0
Bacitracin	2.0 to 20.0 mg.	0
Terramycin	10.0 to 60.0 mg.	0
Chloromycetin	10.0 to 60.0 mg.	0
Sulfanilamide	0.1 to 0.2 mg.	0
Sulfathiazole	0.1 to 0.2 mg.	0
Sulfaguanidine	0.1 to 0.2 mg.	0
Sulfathalidine	0.1 to 0.2 mg.	0
Sulfasuxidine	0.1 to 0.2 mg.	0
Sulfadiazine	0.1 to 0.2 mg.	slight (1st week)
Sodium sulfathiazole	15.0 to 20.0 mg.	0
Sodium sulfamethazine	0.25 Gm.	0
Bismarsen	0.1 Gm.	marked
Mapharsen	0.06 Gm.	0
	0.1 Gm.	moderate
	0.3 Gm.	marked
Merthiolate (aqueous)	1 : 10,000 to 1 : 1,000	marked

The first two were injected into the hardened areas of affected glands with a small-gauge, 2-in. needle, using 0.6 Gm. of mapharsen and 0.4 Gm. of bismarsen each in 2.0 ml. of the aqueous diluent that is supplied in sterile ampules with these agents. Both drugs proved to be rather irritating when given in this manner, and an exacerbation of symptoms resembling those of a Herxheimer reaction in syphilis followed. Four cows received between four and nine treatments each with one or both of these drugs. Milk samples collected from 2 of these cows a month later were free of the Cryptococcus.

Both of these animals had suffered from comparatively mild clinical symptoms some weeks previous to treatment. One of the others was a moderately severe case.

The merthiolate (aqueous) was infused into affected glands in 30.0-ml. amounts of a 1 : 5000 solution. This treatment did not appear to be excessively irritating to the udder tissues, although for a few days after treatment the milk was discolored and rather watery.

Daily treatments were given for four consecutive days to 11 quarters on 7 animals, and the series repeated five days later in the case of 3 of them. These cows were among those which were found to shed the Cryptococcus organisms in their milk for the first time during May and none had been observed to have visible symptoms. Milk samples from all 7 collected from sixteen days to a month after treatment were free of the infection. It was difficult to assess the value of the treatments, even though they appeared to reduce the time required to eliminate the infection, because clinical symptoms had been absent and many of the untreated animals also ceased to shed the organisms during the same period.

An increasing resistance of the udders to invasion or reduced virulence of the Cryptococcus coupled with the control program and possibly some assistance from treatments and feed changes brought about reductions in the numbers of infected stock and the incidence of new infections, with the result that only a few animals were shedding appreciable numbers of the organisms during September.

Calf Exposure

Examination of the carcasses of 9 of the infected cows failed to demonstrate lesions involving *C. neoformans* in locations other than

[f] Abbott Laboratories.
[g] Parke, Davis & Company.

the udder and associated lymph nodes except in 1 animal.[4] As a further check on the invasiveness of the organism, a 10-week-old calf was exposed by feeding 200 ml. of broth culture in milk, by administering 5 ml. of the same intravenously, and by giving 0.5 ml. subcutaneously in the cervical region. It was impossible to find any lesions thirty-seven days later in any of the tissues, either macroscopically or microscopically. Neither was it possible to obtain recovery of the organisms from the tissues by cultural means. Furthermore, examination of fecal samples repeatedly collected during the week following the feeding of the broth culture failed to reveal the presence of the organisms.[5] These findings would indicate that this infection does not very readily establish itself in the tissues of cattle other than the udder.

The Cryptococcus could not be recovered from a piece of fresh beef, size 4 by 4 by 1 in., that was held at —10 F. for a period of two weeks following injection with a suspension containing 1 million viable organisms per milliliter. This further suggests that beef does not provide a satisfactory environment for the organism and that beef so contaminated may become free of the infection after being held for some days at low temperature.

Discussion

The clinical symptoms of gradually increasing severity for several days, intense fullness of the glands, marked reduction in milk flow, and change to viscid secretions, yet lack of serious manifestations of toxemia, indicated that the changes in the glands differed from those encountered in more usual types of mastitis. Gross and histopathological examinations supported this deduction.[4]

An impression gained through clinical observations that the organisms were growing in the milk and distending the alveoli and ducts to their utmost limits may have been correct in regard to the earlier stages but was not entirely supported by histopathological examinations made of tissues from cases of some weeks' duration. In these, not only were masses of the organisms present but also the tissues were heavily infiltrated with large, foamy, mononuclear phagocytes.[4] Regardless, the clinical picture fitted the pattern that might be expected to accompany the obstructive and destructive efforts usually credited to massive growth in tissues of this organism.

Cryptococcus neoformans does not withstand pasteurization temperatures,[3] a detail that received further confirmation in this study, as the organisms were never recovered from the flash-pasteurized bulk supplies from either the healthy or infected animals even though checked repeatedly. Although there should be no danger to consumers of pasteurized products from herds infected with this organism, the advisability of retaining infected stock in a herd may be questioned because of the possible existence of a health hazard for those working with the animals.

Summary

The udders of 106 animals in a 235-cow Holstein-Friesian dairy herd had become infected during the period of a year with *Cryptococcus neoformans*, the causative agent of human cryptococcosis. The origin of the infection could not be determined. Fifty-five of the infected animals developed visibly abnormal conditions of their udders or milk, and the majority of these had severe attacks. Most of the clinical cases occurred during the earlier months of the outbreak, while infection without symptoms was the rule in later months.

The clinical manifestations varied from the presence of slight abnormalities of the foremilk to very severe attacks, which rendered the animals useless for future production. Severe cases were characterized by extreme distention of the gland and edema of surrounding tissues that gradually developed over a period of one to two weeks and persisted for several more. It was accompanied by marked reduction in milk with a change to a gray-white, highly viscid, and mucoid secretion.

Reasonable success followed the use of generally recommended methods for the control of mastitis, including segregation of infected animals, improved sanitation, and limited treatments, coupled with care in milking machine operation and some changes in feeding practices. It appeared that an increase in the resistance of the herd or a reduction in the virulence of the infection assisted materially in bringing the outbreak under control.

Exposure of a calf to *C. neoformans* by oral, intravenous, and subcutaneous administration failed to produce lesions or establish the infection in it. The organisms could not be recovered from fresh beef which had been inoculated with them and then frozen for two weeks.

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Urinary Glucocorticoids as an Indication of Adrenal Dysfunction in Ketosis of Dairy Cattle

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IT HAS BEEN postulated that ketosis (acetonemia) in dairy cattle has, among other etiological factors, its main origin in a temporary corticoadrenal insufficiency.[4] This view has been suggested by the interpretation of some manifestations of the disturbance such as : hypoglycemia, relatively high glucose tolerance,[2] ketosis, frequent improvement from the administration of corticoadrenal hormones (cortisone),[7] temporary course of the disturbance, and low mortality. No experimental data, however, has been presented to support a significant adrenal dysfunction in ketosis of dairy cattle.

The objective of the research reported here was to conduct a preliminary experiment to determine whether significant corticoadrenal activity, as indicated by the urinary output of glucocorticoid hormones, could be detected in cows suffering from ketosis. This was studied by determining and comparing the urinary glucocorticoids from normal, pregnant, and acetonemic cows.

METHODS

A modification of Venning's[6] method for the biological assay of adrenal glucocorticoids in urine was employed. The urinary extracts were prepared as follows: an aliquot of 4 liters was taken from the 24-hour urine collection from 2 normal, 2 pregnant, and 2 acetonemic Holstein-Friesian cows. This was extracted in the following manner: the urine was first adjusted to a pH of 1 by using sulfuric acid with thymol blue as the indicator. It was then extracted four times with chloroform at a concentration of 15 per cent by volume. The emulsions so formed were broken down by centrifugation, and the chloroform extract was evaporated almost to dryness, under reduced pressure, by use of a water bath maintained at 55 C. or lower. The residue was dissolved in 20 ml. of chloroform, which was then extracted three times with 5 ml. of cold, tenth-normal NaOH and three times with 5 ml. of water. These washings were reëxtracted with chloroform. The combined chloroform fractions obtained were evaporated to a volume of approximately 2 ml. under reduced pressure and finally transferred to a test tube. The remainder of the chloroform was then removed under a stream of nitrogen gas. The dry residue was refrigerated until used in the assays described below.

Male albino mice (Rockefeller colony), weighing 20 to 25 Gm., were used for the biological assay. Two days before adrenalectomy, the former diet of Purina dog chow was replaced by the McCollum lactation diet consisting of 26 per cent protein and 52 per cent carbohydrate. The animals were anesthetized with nembutal,® the dose being 2.5 mg. per mouse. The mice were then bilaterally adrenalectomized through the dorsal approach and placed in a room of relatively constant temperature (74 to 76 F.). During the first postoperative day, the mice were given the McCollum diet with a 0.9 per cent NaCl solution containing 5 per cent glucose as drinking water. On the third postoperative day, the food was removed in the evening and the mice fasted for eighteen hours. On the fourth postoperative day, after completion of the eighteen-hour fasting period, the drinking water was removed, and a total of five injections of cow's urinary extract, as described below, were given to each mouse at one-hour intervals.

The residue of the urinary extract for each cow was dissolved in a minimum of ethyl alcohol and made up to a total volume of 11.2 ml. in such a manner as to give a 10 per cent alcoholic solution; to this, glucose was added to make a 5 per cent solution. Of this solution, 0.15 ml. was injected subcutaneously into each mouse at each of the five injections. Control mice received 0.15 ml. of solvent (5% glucose, 10% alcohol) at each of the five injections. One hour after the fifth injection, the mice were weighed successively and anesthetized with nembutal (1.25 mg. per mouse). The livers of the mice were quickly removed and plunged into 4 ml. of hot KOH in 40-ml. centrifuge tubes. The tubes were heated in a boiling water bath and stirred frequently until

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all the liver tissue was in solution. The glycogen was precipitated by the addition of 5 ml. of 95 per cent alcohol, and the tubes heated in a boiling water bath for ninety seconds, the approximate time required for the solution to begin boiling, and then cooled in ice for twenty minutes. After this period, the tubes were centrifuged at 3,000 revolutions per minute (r.p.m.) for five minutes; the supernatant liquid was poured off and the tubes allowed to drain.

The glycogen was then hydrolyzed after the addition of 5 ml. of normal sulfuric acid by placing the tubes in the autoclave for fifteen minutes at 15 lb. of pressure. The glucose was determined by the Shaffer-Somogyi-Hartmann method[6] for blood sugar. In the accompanying tables, the values are expressed as milligrams of glycogen per 100 Gm. of mouse body weight. The experimental data were treated statistically. The standard error of the mean and significant differences were determined by the usual formulas.

RESULTS

The results of the biological assay of urinary extracts from 2 normal lactating cows, expressed in terms of the liver glycogen deposited in the mice, are given in table 1 (cows A and B). Similarly, the data for 2 pregnant cows (pregnancy approximately 8 months) are given in table 2 (cows C and D). In table 3 will be found the data for 2 cows suffering from acetonemia (cows E and F). All cows were of Holstein-Friesian breeding and weighed from 1,000 to 1,300 lb. in the nonpregnant state. Cows A, B, E, and F were producing approximately 45 lb. of milk per day, while cows C and D were dry at the time of observation. During the attack of ketosis, cows E and F showed a decrease in milk secretion to 35 lb. per day, hypoglycemias of 25 and 26 mg. of glucose per 100 ml. of blood, and ketonemias of 55 and 42 mg. of ketone bodies per 100 ml. of blood, respectively.

TABLE 1—Liver Glycogen Deposition Obtained with Urinary Extracts from Normal Lactating Cows (Cows A and B) in the Adrenalectomized Mouse

No. mice	Urinary extract administered	Solvent	Mean liver glycogen deposition (mg./100 Gm. mouse body weight)*
8	None (control)	5% glucose 10% alcohol	3.03±0.25**
7 (1 died)	From cow A	5% glucose 10% alcohol	13.03±0.94**
6 (2 died)	From cow B	5% glucose 10% alcohol	11.34±1.20**

*As calculated for the total amount of urine obtained during a 24-hour period, having as a basis the results obtained with the urinary extracts from 4 liters of urine.
**Standard error of the mean.

TABLE 2—Liver Glycogen Deposition Obtained with Urinary Extracts from Pregnant Cows (Cows C and D) in the Adrenalectomized Mouse

No. mice	Urinary extract administered	Solvent	Mean liver glycogen deposition (mg./100 Gm. mouse body weight)*
7 (1 died)	None (control)	5% glucose 10% alcohol	3.11±0.95**
7 (1 died)	From cow C	5% glucose 10% alcohol	21.27±1.00**
7 (1 died)	From cow D	5% glucose 10% alcohol	18.93±0.92**

*As calculated for the total amount of urine obtained during a 24-hour period, having as a basis the results obtained with the urinary extracts from 4 liters of urine.
**Standard error of the mean.

Eight mice were employed in testing the urinary extracts of each experimental cow and also as controls in each experiment. Mice which died during the experiment were eliminated from the calculations. The results of the different biological assays of urinary extracts from the aliquots of 4 liters of urine are expressed in the tables as mean liver glycogen deposition in milligrams per 100 Gm. of mouse body weight, after being calculated for the total amount of urine excreted by each cow during a 24-hour period.

From the data reported here, it can be seen that the values for the mean liver glycogen deposition obtained following injections of urinary extracts from acetone-

TABLE 3—Liver Glycogen Deposition Obtained with Urinary Extracts from Acetonemic Cows (Cows E and F) in the Adrenalectomized Mouse

No. mice	Urinary extract administered	Solvent	Mean liver glycogen deposition (mg./100 Gm. mouse body weight)*
7 (1 died)	None (control)	5% glucose 10% alcohol	3.00±0.81**
6 (2 died)	From cow E	5% glucose 10% alcohol	6.30±0.27**
7 (1 died)	From cow F	5% glucose 10% alcohol	5.04±0.19**

*As calculated for the total amount of urine obtained during a 24-hour period, having as a basis the results obtained with the urinary extracts from 4 liters of urine.
**Standard error of the mean.

mic cows, as compared with those obtained from injections of similar extracts from normal lactating cows, are significantly decreased; and, as compared with the values obtained from urine extracts from pregnant cows, are decreased to a greater extent (table 4).

DISCUSSION

Reinecke and Kendall,[5] and Dobriner[1] have shown that the livers of fasted, adrenalectomized rats and mice, respectively, are quickly depleted of glycogen and that the administration of corticoadrenal hormones leads to the deposition of large amounts of liver glycogen. In the experiment reported here, the larger quantities of liver glycogen observed in the fasted, adrenalectomized mice which had received injections of cow's urinary extract, as compared with the controls, may be attributed to a corticoadrenal hormone activity present in the urine of the cows.

TABLE 4—Significance of Difference Between the Results Obtained

Table 1 (Normal lactating cows)	Table 2 (Pregnant cows)	Significance of difference ('t'*)
Cow A	Cow C	5.8
Cow B	Cow D	5.0

Table 1 (Normal lactating cows)	Table 3 (Actonemic cows)	Significance of difference ('t')
Cow A	Cow E	6.8
Cow B	Cow F	5.2

*'t' is the significance factor of Fisher. A 't' factor greater than 2 indicates a significant difference.

According to Venning *et al.*,[8] the use of glucose in the solution administered to the mice during the assay has no appreciable effect on the liver glycogen deposition but has the advantage of increasing the sensitivity of the assay. Furthermore, Long *et al.*[3] have shown that the absolute increase in the carbohydrate stores of fasted animals receiving corticoadrenal hormones occurs at the expense of the protein stores of the body.

The significantly different increases of glycogen deposition obtained in mice with the administration of a constant amount of urinary extract from pregnant, normal lactating, and acetonemic cows indirectly indicates decreasing amounts of corticoadrenal activity. The results seem to be in agreement with the different physiological or functional states of the animals tested. Pregnant cows, which presumably are under an intense physiological stress, show an increase in corticoadrenal activity as compared to the normal lactating cows. Acetonemic cows, in agreement with a previous hypothesis postulated,[4] show a decreased corticoadrenal activity as compared to both normal lactating and pregnant cows.

These results appear to be specifically suggestive when we consider that the factors of age, breed, weight, and milk secretion were similar in the cows chosen for these experiments.

SUMMARY

A biological assay of urinary glucocorticoids, as an indirect method of determining the level of corticoadrenal activity in normal lactating, pregnant, and acetonemic cows, was made. Pregnant cows showed an increase and acetonemic cows showed a decrease of corticoadrenal activity, as compared to that of normal lactating cows in this preliminary experiment.

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A Study of Moraxella Bovis and Its Relation to Bovine Keratitis

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KERATITIS OF AN infectious nature in cattle has been studied in this country and throughout the world. Foreign workers[9, 53, 20, 15, 60, 42] have attributed causal relationship of a conjunctivitis and keratitis of cattle to a rickettsial-like organism, *Colesiota conjunctivae* (*Richettsia conjunctivae*),* whereas American workers[2, 33, 5, 51] have incriminated a bacterial agent, *Moraxella bovis* (*Hemophilus bovis*).*

It is granted that ophthalmitis is a part of the symptomatology of various diseases of cattle and may be due to causes other than the bacterial agent described in this paper. Nutrition may play a part, as is evidenced by the various reports on vitamin A deficiency, wherein an inflammation of the eyes, with resulting corneal opacities and ulcerations, has been encountered.[3, 29, 35, 57] Likewise, allergy and parasites may be factors in the causation of ocular pathology.

A complete understanding of a single disease entity in all its complexity is more than an individual can hope to achieve. However, this study was instigated in behalf of the livestock industry upon whose resources this disease is a continual drain. The initial isolation of *Mor. bovis* was made in a herd of Kansas dairy cattle affected with keratitis of undetermined origin. The symptomatology, in general, consisted of a conjunctivitis, photophobia, impaired vision, and lacrimation of a thin, clear, watery fluid which occasionally was somewhat mucopurlent. The lesions were unilateral or bilateral and accompanied by a varied degree of milky or cloudy discoloration of the cornea with peripheral vascularization, followed, in some instances, by a more or less centrally located area of ulceration.

Portion of a thesis as partial fulfillment of the requirements for the degree of doctor of philosophy in bacteriology at Kansas State College.

Contribution No. 269, Department of Bacteriology, Kansas Agricultural Experiment Station, Manhattan.

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*Terminology formerly used in Bergey's "Manual of Determinative Bacteriology," 5th ed., William & Wilkins Company, Baltimore, Md., 1939.

REVIEW OF LITERATURE

From the available literature, the first report on bovine keratitis was that by Billings,[8] who reported this condition in 1889 and stated that it was not a new disease so far as the United States was concerned. In his report, his observations were confined to a herd of dairy cattle in the vicinity of Lincoln, Neb., during the summer and fall of 1888. He recognized the presence of thin, short bacilli with rounded ends in sections of the cornea, but was unsuccessful in reproducing the disease by transferring the organisms from the eyes of diseased cattle to healthy cattle. His experiments with rabbits were likewise negative.

Allen,[2] of Canada, reported in 1919 that infectious bovine keratitis had a world-wide distribution and that, although there was a striking similarity in the symptomatology in widely separated portions of the world, the disease might be caused by the same organism. In the outbreak which occurred in Quebec, Allen was able to demonstrate a short, thick, gram-negative diplobacillus. Before attempting to cultivate the organism, he reproduced the pathological eye condition by direct transfer in a healthy heifer and a young bull. His initial efforts to cultivate the organism on agar resulted in failure; however, he successfully isolated the diplobacillus by means of Loeffler's blood serum. It was noted that the organism was pleomorphic and resembled somewhat the organism associated with infectious conjunctivitis in man, as described by Morax[44] in 1896 and Axenfeld[4] in 1897. Inoculation experiments conducted on rabbits and guinea pigs met with negative results.[2]

Mitter,[43] of India, reported in 1915 on his investigation into an epidemic of contagious ophthalmia among conservancy cattle. According to his findings, the outbreak appeared to be caused mainly by *Micrococcus lanceolatus* and partly by the bacillus of Morax-Axenfeld. He was unsuccessful in his attempts to reproduce the disease in experimental animals and stated that it was highly probable that the outbreak originated by infection from human beings.

A translation by Kappeyney and Ward[36] was made in 1917 of an article by Poels,[49] who conducted an investigation of an ophthalmia of cattle in Holland. Poels dissected out a portion of diseased cornea and recovered *Bacillus pyogenes*. While he could not reproduce the disease by inoculating the cornea with *B. pyo-*

genes, he reproduced a typical case of keratitis by injecting the organisms between the layers of the cornea. Poels concluded that *B. pyogenes* was the true cause of keratitis infections in Holland.

Jones and Little[23] reported in 1923 on their observations of 24 cases of acute ophthalmia of cattle. From each case, they recovered a characteristic gram-negative, proteolytic, and hemolytic diplobacillus. Five of the 10 cows reëxamined after three or four months continued to carry the diplobacillus. Four normal cattle, when inoculated beneath the eyelids with bouillon suspensions of pure cultures, developed characteristic inflammations. Laboratory animals were refractory to suspensions of exudate made from the eyes of cattle and also to heavy suspensions from freshly isolated cultures.

Jones and Little[24] later reported (in 1924) on the transmission and treatment of infectious ophthalmia of cattle. Bacteria were recovered in the early stages of the disease from the nasal passages of experimentally inoculated animals. In a large number of well-established cases, bacteriological findings were negative. They found that the characteristic diplobacillus was rapidly destroyed in the digestive tract of flies and that the organism lived only three hours on the external surfaces of flies. In their experiments, they failed to show that the disease could be transmitted by flies, but the writers suggested that the methods employed were probably at fault. They found that 2.5 per cent zinc sulfate was more effective than 20 per cent argyrol in promptly ridding the diseased eyes of the diplobacilli.

Hauduroy *et al.*[30] in 1937 classified the diplobacillus isolated by Jones and Little[23] as *H. bovis* (Jones and Little). A. Lwoff[40] in 1939 included the organism isolated by Jones and Little in the new genus Moraxella. Bergey's "Manual of Determinative Bacteriology,"[10] likewise, follows this classification.

Farley[21] in 1941 attempted to reproduce keratitis in 38 calves and 4 cows by means of filtrates of virulent eye secretions but was unsuccessful. Approximately, 70 per cent of these animals were proved to be susceptible when exposed to virulent eye secretions. He experimentally infected numerous cattle with unfiltered virulent eye secretions of diseased cattle. He found that some calves were naturally resistant to infection and could not be caused to contract the disease, even though they were repeatedly exposed for a long time. Three types of the disease were demonstrated in calves: the mild, acute, and chronic types. Farley was unable to reinfect those calves which had recovered. He stated that keratitis of cattle is probably a localized infection and that calves recovering from the chronic type may prove a source of infection for seven months or longer. He made numerous attempts to transmit the disease from sheep to cattle and from cattle to sheep, but the infection proved to be species specific in all cases. *Escherichia coli*, *Corynebacterium pyogenes*, streptococci, staphylococci, and *Pasteurella boviseptica* were isolated from the eyes of calves affected with keratitis and did not reproduce the disease in calves held under laboratory conditions. Farley[21] stated that studies in the laboratory have proved fairly conclusively that the etiological agent of keratitis does not pass through filter candles of medium and fine density, indicating that the causative factor was of a bacterial nature. Farley *et al.*[35] made a preliminary report in 1950 on infectious keratitis of cattle, wherein they isolated *H. bovis* from active field cases but were unable to reproduce the disease in 5 normal calves.

Baldwin[5] reported in 1945 that he found *H. bovis* in 93 of 112 infected eyes of cattle and was able to induce an infection indistinguishable from infectious keratitis in one or both eyes of 12 of 15 animals. The organism was not found in either eye of 20 normal cattle. He found that mice, guinea pigs, and rabbits were not susceptible to *H. bovis*.

Reid and Anigstein[51] reported in 1945 on their investigation of infectious keratoconjunctivitis of cattle on the Gulf Coast of Texas. In their microscopic studies of the conjunctiva of affected cattle, they found intracellular and extracellular minute bodies which morphologically resembled rickettsias, but they could produce no evidence of a causal relationship. The disease was easily produced experimentally in calves by contact with the eye or nasal exudates, but the bacteria-free filtrates were nonvirulent. Reid and Anigstein[51] stated:

> Since the disease is transmitted from infected to susceptible animals by direct contact, attempts were made to grow the causative agent on artificial media from the exudate. Scrapings were made from the conjunctiva of the infected eye and plated on nutrient agar. The following organisms were isolated: *Escherichia coli*, *Bacillus pyogenes*, *Bacillus subtilis*, Pasteurella and Staphylococcus. When these organisms were placed on the conjunctiva of normal cattle no evidence of the disease developed. In addition, suitable media consisting of embryonic mouse brain and ox serum ultra-filtrate, described by Sanders (1942), were used in an attempt to cultivate the causative agent.

In several instances, bacteria-free conjunctival scrapings from acute cases were obtained and inoculated into developing eggs. Small coccoid bodies, scattered or in groups, were demonstrated in Giemsa-stained smears made from injected egg yolk sacs. No conclusions could be drawn as to the nature of these bodies in that attempts to infect calves' eyes with bacteriological sterile material gave negative results. These authors[51] stated:

Systematic investigation of the bacterial flora of infectious keratoconjunctivitis revealed, in addition to various coccal organisms cultivated on ordinary media, a hemophilic and hemolytic bacillus which requires blood or serum for growth. Experimental keratoconjunctivitis in cattle, sheep, and goats was produced by young cultures of this bacillus. In addition, agglutinins specific for this organism were found in the sera of cattle convalescent from the natural or experimental infection.

They considered the hemophilic and hemolytic bacillus to be closely related, if not identical, to *H. bovis*. On the basis of their work, wherein they reported that 1 sheep and 2 goats proved susceptible to a hemolytic diplobacillus, Reid and Anigstein[24] suggested that the name *Hemophilus ruminantium* would seem more appropriate should the same organism be found in spontaneous keratitis of sheep and goats.

Roderick[52] recognized an eye condition of sheep as an infectious disease, as did Farley[23] and Baldwin.[5] Baldwin stated that sheep are susceptible to an eye disease which is clinically similar to bovine infectious keratitis. He further stated that the failure of sheep to react to inoculations of either virulent eye secretions of cattle or of *H. bovis* definitely indicated that the eye disease in sheep is caused by some other agent. Keratitis of sheep is cosmopolitan in distribution and was reported in the United States by Johnson [22] and Boughton and Hardy[11] as being caused by a rickettsial-like organism.

Coles[15] reported in 1936 the observation of a rickettsial-like organism in the conjunctival epithelium of cattle after having made a similar finding in sheep.[12] He provisionally proposed the name *R. conjunctivae bovis* for it. The rickettsial-like organism demonstrated and described by Coles[12, 14, 16, 17] in sheep, goats, chickens, and hogs, in addition to cattle, has since been renamed *C. conjunctivae* by Rake[39] and placed in the family Chlamydozoaceae.

Other foreign writers[19, 21, 27, 41, 46, 48, 58] have, likewise, identified rickettsial-like or cell-inclusion bodies in the conjunctival epithelium of sheep or goats. Cultural work of a bacteriological nature apparently was not carried out in the investigational work herein cited. Blakemore[9] stated:

> It seems probable that a number of specific infective conditions affecting the superficial structure of the eye may occur in bovines but, as indicated by the work of Coles, one form of the disease is characterized by the presence of intracellular inclusion bodies in the conjunctiva. The same condition has now been recognized in this country. Whether or not it is identical with contagious ophthalmia referred to in American literature, and attributed to *Hemophilus bovis* infection Baldwin, 1945) must await further investigation but the severe cases observed in outbreak 2 would appear to be very similar clinically.

Morphological, Cultural, and Physiological Characteristics of Moraxella Bovis.—Organisms isolated from the conjunctival sacs of Kansas cattle affected with keratitis were identified by means of Bergey's manual[10] as *Mor. bovis*. These organisms were discrete, short, plumb diplobacilli with rounded ends, measuring 0.5 to 1.0 μ in width and varying in length from 1.5 to 2 μ (plate 1). The bacteria were fairly uniform in 24- or 48-hour cultures. Pleomorphism was very pronounced in older cultures. Cells occurred singly, in short chains of 4 to 8 bacilli or coccobacilli, and occasionally occurred as threadlike filaments or large coccoid forms. They did not form endospores and were nonmotile in hanging-drop preparations. Capsules were present in stained India ink preparations. Cells did not stain acid fast. With Hucker's modification of Gram's method, the cells stained negatively.

Surface colonies on proteose peptone agar plates, enriched with 5 per cent defibrinated bovine blood, were circular, umbonate, entire, smooth, translucent, and greyish white in color. Growth in twenty-four hours was abundant. The colonies measured 1 to 2 mm. in width. After forty-eight hours, the colonies reached a diameter of 3 to 5 mm. and a hemolytic zone with a width of 2 to 3 mm. Primary isolations of *Mor. bovis* were made, also, on plain proteose peptone agar, the colony morphology being similar to those initially isolated on proteose peptone agar to which had been added 5 per cent defibrinated bovine blood. The use of 2, 3, 5-triphenyltetrazolium chloride* (.0025%) in proteose peptone agar did not reveal any color pattern differences in the colonies of eight cultures recently isolated from different outbreaks. Deep colonies were ovoid and biconvex in shape. After forty-eight hours' incubation in proteose peptone broth, the turbidity was slight with considerable sediment formed which, when agitated, broke up into coarse particles.

Cultures were maintained at room temperature in screw top test tubes without transfer for the following periods: four months on proteose peptone agar, two and one-half months on Loeffler's serum agar, and four months on proteose peptone agar enriched with 5 per cent defibrinated bovine blood and overlaid with sterile mineral oil. Cultures tended to die out within four to ten days when placed in the refrigerator at 4 C. Fair to good growth of *Mor. bovis* was present on nutrient agar and tryptose agar. Excellent growth was present on stock culture agar, proteose peptone agar, and proteose No. 3 agar, and also on plates of proteose peptone agar and stock culture agar which were enriched with 5 per cent defibrinated normal bovine blood, 5 per cent whole bovine blood from an acute case, and 5

*Referred to hereafter as tetrazolium.

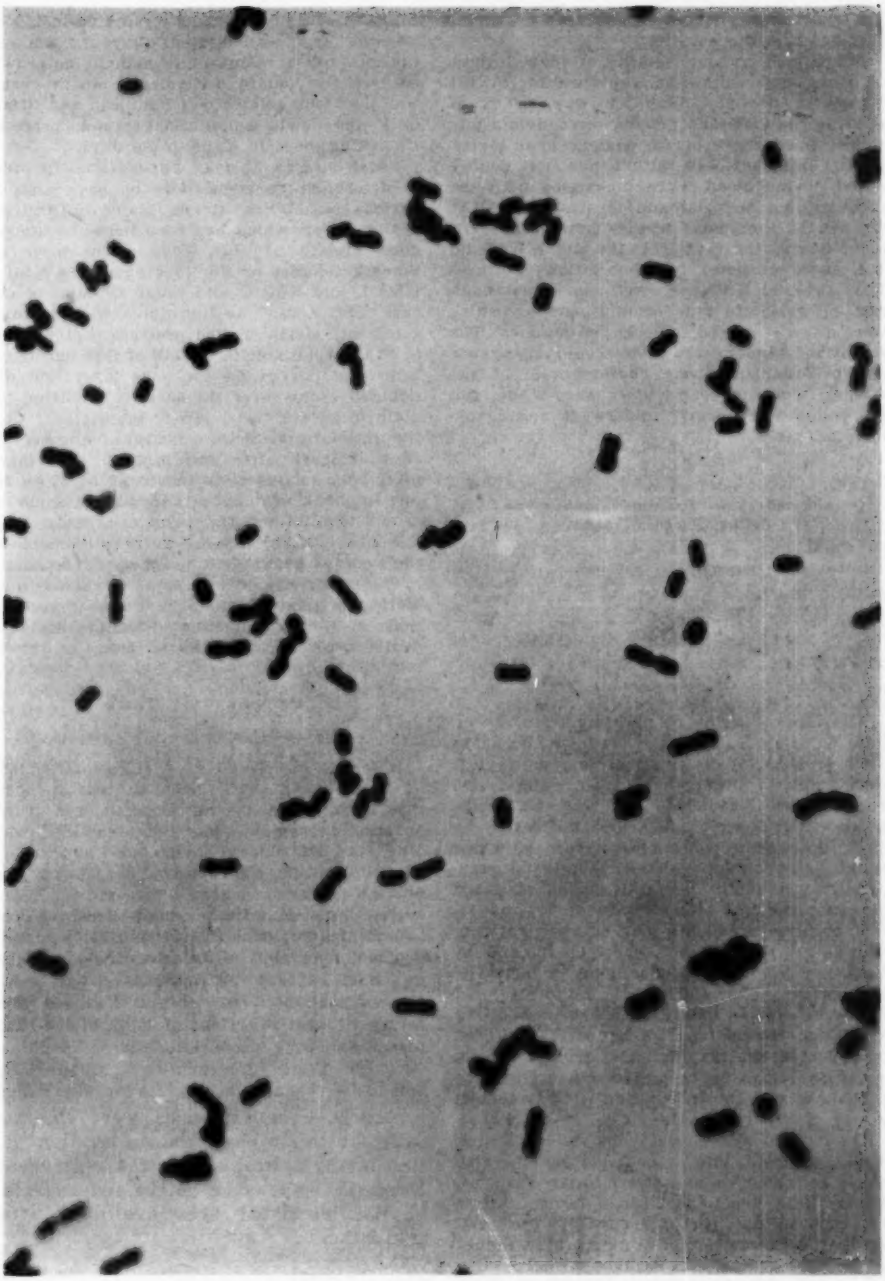

Plate 1—Moraxella bovis (24-hour culture) stained by Gram's technique. Note the typical diplobacillus arrangement. x 2000.

per cent whole bovine blood from a chronic carrier of bovine keratitis.

Table 1 shows the viability of four cultures of *Mor. bovis* following lyophilization by the Cryochem process. Viability was tested by opening the vials and placing the contents into tubes of saline. Proteose peptone agar plates, having been enriched with 5 per cent defibrinated bovine blood, were inoculated with the reconstituted material and incubated at 37 C. Growth of *Mor. bovis* was evident after forty-eight hours. Cultures KSC 409 and KSC 7 did not show evidence of dissociation; cultures KSC 494 and KSC 8 showed numerous rough and intermediate type colonies, among which were a few scattered smooth type colonies. The colony morphology and microscopic appearance of the organisms were characteristic of the species. Subsequent transfers were made, and the colonies frequently underwent morphological changes.

TABLE 1—Survival Time of Various Cultures of Moraxella Bovis Following Lyophilization by the Cryochem Process

Culture No. *Mor. bovis*	Date lyophilized	Held after lyophilization	Test for viability
KSC 494	10-11-50	8 mo.	+
KSC 409	10-11-50	8 mo.	+
KSC 7	5-7-50	1 mo.	+
KSC 8	5-7-50	1 mo.	+

+ = viable.

After four months of multiple transfers on solid culture mediums, the organism began to show evidence of dissociation from the typical smooth forms into rough, intermediate, and dwarf forms. Mucoid variants were not recognized. After forty-eight hours' incubation at 37 C., the rough colonies had a very irregular margin; the surface was very flat and occasionally granular. The fermentation reactions were not changed. Gram stain of 24- and 48-hour cultures revealed a marked pleomorphism with large, round forms, short chain, and irregular rods being predominant among the diplobacilli. The intermediate forms had slightly irregular margins, the surfaces being convex or umbonate. The dwarflike colonies were 1 to 2 mm. in diameter and did not increase in size even though multiple transfers were made and the inoculation time prolonged beyond forty-eight hours.

The culture phases were not stable. Rough phases reversed into smooth forms and vice versa. Intermediate and dwarf forms, likewise, changed into smooth and rough forms. Old or senescent cultures (5 to 7 days) showed evidence of the formation of minute protuberances or papillae. Daughter colonies were also observed.

As for physiological characteristics, indole was not produced; nitrates were not reduced to nitrites; no acid or gas was noted in xylose, glucose, fructose, lactose, sucrose, maltose, inulin, dextrin, mannitol, or salicin; no growth occurred on potato; coagulated bovine serum was liquified; gelatin was liquified; and litmus milk was slowly peptonized, complete proteolysis taking place in eight to ten days.

Sensitivity to Various Antibiotics.—In order to determine the sensitivity of *Mor. bovis* to various antibictics, sterile plates of proteose peptone agar, which had been found to support good growth of *Mor. bovis*, were prepared. Smooth colonies of *Mor. bovis* cultures KSC 2, KSC 4, and KSC 6, and rough colonies of culture KSC 8 used as inoculums were incorporated into sterile melted proteose peptone agar at 45 C. Approximately 5 ml. of this inoculated agar was poured on the base layer and distributed evenly over the surface by tilting the plate to give a "seed layer" inoculation; then the antibiotic discs were placed on the surface of this layer after solidification had taken place. The plates were incubated at 37 C. for forty-eight hours and examined for zones of growth inhibition. Duplicate tests were made for each culture, and the average diameter of the zones of growth inhibition were determined.

The sensitivity of *Mor. bovis* to various antibiotics is given in table 2. Under the conditions of the experiment, chloramphenicol and penicillin produced the larger zones of growth inhibition.

PATHOGENICITY TESTS

PART A—SMALL EXPERIMENTAL ANIMALS

Materials and Methods.—A culture (KSC 409) of *Mor. bovis* recently isolated from an acute case of infecticus bovine keratitis was grown on proteose peptone agar plates enriched with 5 per cent defibrinated bovine blood and washed off with saline. The heavy suspension was filtered through a cotton filter into an Erlenmeyer flask containing glass beads, shaken, and diluted to turbidity No. 1 of McFarland's nephelometer. One milliliter of this suspension was instilled into the conjunctival sac of the eyes of each of 2 rabbits and 2 guinea pigs. Intraperitoneal injections of 1 ml. of the same suspension were made into each of 2 guinea pigs and 2 ml. into each of 2 rabbits. The animals were placed in cubicles for observation.

Results.—These animals were observed daily for thirty days, and no reaction was seen in any animal used in the experiment. *Moraxella bovis* could not be demonstrated in the inoculated eyes five days after instillation.

PART B—SHEEP

Materials and Methods.—The conjunctival sacs of both eyes of 2 aged ewes and 6 young

TABLE 2—Sensitivity of Moraxella Bovis to Antibiotics

Type of antibiotic disc	Amounts	Zones of inhibition expressed in millimeters: Culture No. KSC 2	Culture No. KSC 4	Culture No. KSC 6	Culture No. KSC 8
	(units)				
Bacitracin	2.0	2.2	2.0	1.8	2.4
	10.0	2.9	2.4	2.6	2.6
	20.0	3.6	2.8	3.0	3.3
Chloramphenicol	10.0	3.2	3.5	3.5	3.8
	30.0	4.4	3.6	4.2	4.0
	60.0	4.6	4.2	4.2	4.4
Penicillin	0.5	3.4	3.2	3.5	3.3
	1.0	3.6	3.4	4.0	3.4
	10.0	4.0	4.5	5.0	4.6
	(micrograms)				
Dihydrostreptomycin	0.5	1.0	0.9	1.0	1.8
	1.0	1.4	1.2	1.5	2.0
	10.0	2.3	2.1	2.2	3.0
Terramycin	10.0	2.5	2.5	3.4	3.7
	30.0	3.3	2.9	3.9	4.9
	60.0	3.7	3.4	4.0	5.1

sheep were checked for *Mor. bovis* and found to be negative. The conjunctival sacs of both eyes of each of these animals were inoculated with a culture of *Mor. bovis* (culture KSC 409) prepared in the same manner as before (Part A).

These sheep were inoculated a second time three weeks later with a suspension of *Mor. bovis* (*H. bovis* (Ohio)) made from smooth colonies of strains 2, 9, and 14, which were obtained through the courtesy of Dr. E. M. Baldwin.*

Results.—None of these animals reacted to the instillation of *Mor. bovis*. The organism could not be demonstrated in the eyes of any of the sheep five days following the inoculations.

PART C—CATTLE

Materials and Methods.—Four calves of mixed breeds with a history of not having shown any evidence of infectious keratitis were selected for this experiment. Bacteriological examinations of the conjunctivas were negative for *Mor. bovis*. The animals were confined to fly-proof stalls.

The inoculum of *Mor. bovis* consisted of culture KSC 1 obtained from an active field case of infectious bovine keratitis in a dairy cow. This culture was transferred seven times on proteose peptone agar enriched with 5 per cent defibrinated bovine blood. The culture was suspended in saline, shaken, and diluted to turbidity No. 1 of McFarland's nephelometer, and 1 ml. instilled into the conjunctival sacs of both eyes of each animal. The eyelids were held closed for several minutes and then massaged.

Results.—The 4 experimental calves developed typical lesions of bovine keratitis. The clinical symptoms and the incubation periods are given in table 3.

Moraxella bovis was recovered from the eyes of each of these calves. They were then placed in a large lot with 3 adult cattle. Two of the adult cows showed an acute ophthalmitis in the left eye only within three weeks; the third remained normal.

All animals were checked a year later for incidence of the chronic carrier stage. All were negative with the exception of calf 2; the organism (KSC 494) recovered from this chronic carrier was sent to the American Type Culture Collection, Washington, D. C., where it was given the accession number 10900. Another culture of *Mor. bovis*, isolated from an acute case of infectious keratitis in a calf, has been added to the American Type Culture Collection as culture KSC 7, accession number 10901.

The 4 calves listed in table 3 and the 3 adult cattle mentioned were exposed to heavy suspensions of culture KSC 8—a smooth culture recently isolated from an acute case of bovine keratitis. Both eyes of each animal were inoculated and observed for over six weeks. No clinical symptoms of keratitis were developed.

ISOLATION OF *MORAXELLA BOVIS* FROM CLINICAL CASES OF BOVINE KERATITIS

Materials and Methods.—One dairy herd of 9 Holstein-Friesian cows and 63 beef cattle involving eight different range herds were subjected to cultural examination. On some of the ranches where a squeeze chute was not readily available, the animals were driven to one

*The Corn States Serum Company, Omaha 8, Neb.

corner of the pasture and secured with the aid of a lasso. The head of each animal was restrained and the eyelids were forced open. A previously prepared sterile swab was then placed upon the conjunctiva in the region of the membrana nictatans and removed after partially turning the swab several times in order to facilitate saturation. In addition, swabs were made of the nasal mucosa of 5 acutely affected beef cattle. The tip of the swab was used to inoculate previously prepared ster-

TABLE 3—Pathogenicity of Moraxella Bovis for Cattle

Calf No.	Clinical reaction		Incubation period (days)
	Right eye	Left eye	
1	Acute ophthalmitis Completely blind	Mild ophthalmitis Recovery	17
2	Acute ophthalmitis Recovery	Acute ophthalmitis Recovery	15
3	Acute ophthalmitis Opaque spot Recovery	Unaffected	21
4	Acute ophthalmitis Recovery	Unaffected	20

ile plates of proteose peptone agar enriched with 5 per cent defibrinated bovine blood. The cultures were incubated for twenty-four hours at 37 C. and examined at 24- and 48-hour intervals. No attempt was made to determine how many animals had a bilateral or unilateral infection or how many unilateral infections developed into bilateral infections. Blood specimens (5 ml.) were taken aseptically from each of 4 acute cases of bovine keratitis and were incorporated at the rate of 5 cc. per 100 cc. of melted proteose peptone agar and plated.

Results.—Colonies having the cultural and morphological characteristics of *Mor. bovis* were isolated from 69 of the 72 animals. The colonies of micrococci and streptococci, both hemolytic and nonhemolytic types, were commonly encountered on the culture plates along with colonies of *Mor. bovis*, which were very prominent and usually in the majority. Occasionally, colonies of Neisseria, Pasteurella, Escherichia, and diphtheroids were identified. Pure cultures of *Mor. bovis* were isolated on several plates. Findings were negative in the case of 3 beef cattle, one of which might have been the result of excessive overgrowth of saprophytic bacteria. Nasal swabs from 3 of the 5 animals were positive for *Mor. bovis*. Blood specimens taken from 4 acute cases of bovine keratitis were negative for presence of the organism.

STUDY OF THE CONJUNCTIVAL EPITHELICAL CELLS AND ATTEMPTED ISOLATION OF *Moraxella Bovis* FROM THE EYES OF NORMAL CATTLE AND SHEEP AND THOSE AFFECTED WITH KERATITIS

Materials and Methods. — Scrapings were made of the conjunctival epithelium of normal cattle and sheep and also cattle and sheep affected with an acute keratitis. The epithelial cells were obtained by everting the upper or lower eyelids of the animals and gently scraping the conjunctivas with a scalpel, care being taken to avoid hemorrhage. The cells were then transferred from the knife by smearing them over a clean glass slide. The slide was dried quickly and fixed in methyl alcohol for three to five minutes, and then stained with 1 ml. of Giemsa's stain in 40 ml. of buffer solution placed in a Koplin jar. After two to four hours' staining, the slides were removed, washed with tap water, dried, and examined under oil immersion.

From the aforementioned animals, conjunctival swabs were taken and plated after the manner described previously.

Results.—The epithelial cells of the conjunctivas taken from the normal and diseased eyes of cattle and sheep occasionally showed the presence of rod-shaped or coccoid bacteria and pigmented particles which were identified as melanin. The melanin granules, while normally golden brown, dark brown, or black, often had a bluish or greenish tinge when stained with Giemsa's stain and resembled pleuropneumonia-like organisms (PPLO) or rickettsial-like organisms (RLO). When decolorized with acid alcohol, the melanin granules reverted to their dark brown color.

In the eyes of some apparently normal and diseased sheep, intracellular organisms, measuring 0.6 to 1.5 by 0.6 to 2.0 μ, blue in appearance, gram negative, being generally round or ovoid, and distributed irregularly through the cytoplasm of the conjunctival epithelial cells, were noted. These organisms of an unknown nature were not identified in any of the conjunctival epithelial cells of normal cattle or cattle affected with keratitis.

Intracellular and extracellular organisms of a PPLO or RLO nature were not present in scrapings made from the eyes of cattle affected with keratitis and from which *Mor. bovis* was isolated (table 4). Occasionally, diplobacilli could be demonstrated. The conjunctival epithelial cells of sheep affected with keratitis contained pleomorphic organisms (table 4) which were intracellular and extracellular, round, oval, or

TABLE 4—Examination Findings of Conjunctival Scrapings and Conjunctival Swabs

Animals	No. animals	Conjunctival scrapings			Conjunctival swabs
		Positive for melanin	Presence of PPLO* or RLO**	Presence of unknown intracellular organisms	Isolation of *Mor. bovis*
Normal calves	11	7	0	0	0
Normal cows	25	21	0	0	0
Diseased calves***	8	5	0	0	8
Diseased cows***	15	11	0	0	15
Normal sheep	15	12	0	4	0
Diseased sheep	55	49	55	23	0
Diseased lambs	12	9	0	0	0

*PPLO = pleuropneumonia-like organisms; **RLO = rickettsial-like organisms; ***These animals are in addition to those reported previously.

rod-shaped bodies measuring 0.3 to 0.8 by 0.3 to 1.0 μ and stained a pale blue. Some of the PPLO or RLO stained evenly; others tended to have a pale central area. Many of the organisms exhibited a halo effect—an empty space 0.3 to 0.5 μ in width. During the preparation of the slides, some of the cell walls apparently were broken down, and many organisms were dispersed over the slides along with debris. Numerous neutrophils, a few erythrocytes, and occasionally bacteria were observed. These organisms were thought to be indistinguishable from the microörganisms of the pleuropneumonia group and also to be identical with *C. conjunctivae*[50] (plate 2).

Moraxella bovis was not isolated from either eye of 36 normal cattle, 15 normal sheep, and 62 sheep with an acute keratitis. The bacillus was isolated from 23 cattle with an acute keratitis.

DISCUSSION

The genus Moraxella was introduced by Lwoff[40] in 1939 and was incorporated in the latest edition of Bergey's manual.[10] The organisms in the genus Moraxella occur predominantly as diplobacilli in recently isolated cultures, whereas the organisms in the genus Hemophilus predominantly occur singly. Inasmuch as Bergey classifies the organism which has been isolated from cases of acute ophthalmia of cattle (pinkeye) as *Mor. bovis*, it was thought to be appropriate to use this nomenclature. It is granted that there are grounds for improvement in classification of the tribe Hemophileae.

The morphology and cultural characteristics of *Mor. bovis* as determined by this study were comparable to the findings of Allen,[2] Jones and Little,[33] Baldwin,[5] Reid and Anigstein,[51] and Farley *et al.*[25] In this investigation, initial isolations of the organism were made on proteose peptone agar in addition to proteose peptone agar enriched with 5 per cent bovine, ovine, or equine blood. It was also determined that subsequent subcultures could be made on proteose peptone agar without the use of added blood, ascitic fluid, other body fluids, or sterile, unheated plant tissue (potato). The reaction on litmus milk with the young cultures differed from that reported by Jones and Little[33] and Reid and Anigstein,[51] wherein they described litmus milk, after seven to ten days' incubation, as exhibiting three distinct, plainly visible zones—an upper, liquid zone of a deep blue color; an intermediate, lavender-colored zone; and, at the bottom of the tube, a light lavender-colored zone of coagulated casein. Litmus milk, when inoculated with various cultures of *Mor. bovis* isolated from cattle in Kansas, was slowly peptonized and complete proteolysis took place in eight to ten days. A scanty amount of casein precipitate was present at the bottom of the tube. Distinct zones, as described by the aforementioned investigators,[33, 51] were not apparent in any of the eight cultures of *Mor. bovis* isolated from eight herds of cattle.

Tetrazolium has been recognized recently as an aid in various bacteriological laboratory procedures.[31, 38, 39, 61] This salt was used in an attempt to detect variation in strains of recently isolated cultures of *Mor. bovis* but, of the eight cultures from different outbreaks, none showed any apparent detectable variance of color pattern.

Practicable apparatus and procedure for the preservation of biological products have been described in detail by Flosdorf and Mudd[27, 28] and Mudd *et al.*[45] Rapid desiccation, under high vacuum, of biological material in the frozen state, such as microörganisms, serums, and viruses, has been found an extremely effective method of

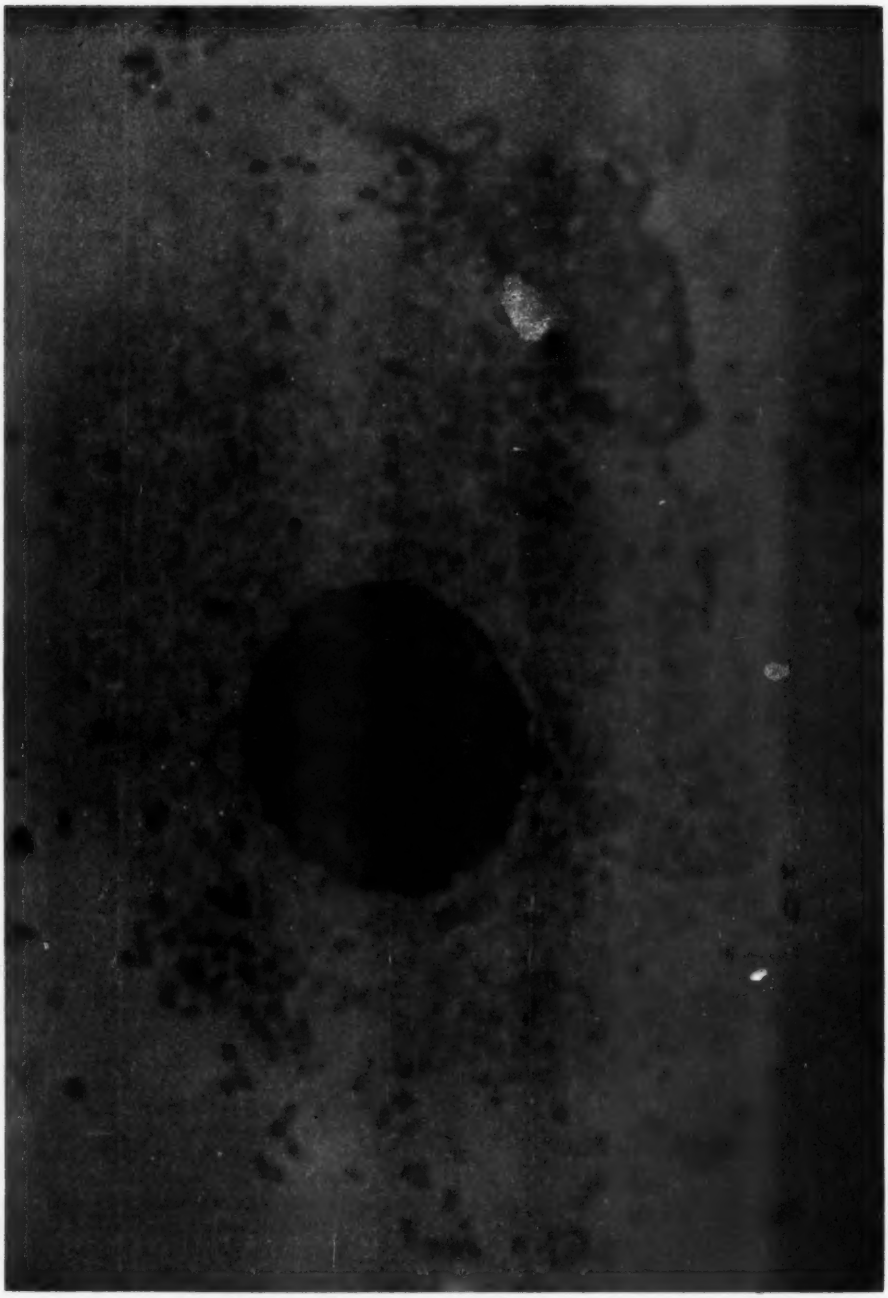

Plate 2—Conjunctival epithelial cell of a sheep affected with acute keratitis. Note the cytoplasm heavily infected with pleuropneumonia-like organisms. Giemsa stain. x 3000.

preserving such material and is used widely in varous laboratories.[59] *Moraxella bovis*, which is a fickle organism in its cultural requirements, was lyophilized and found viable after at least eight months. Baldwin[6] has made use of lyophilization as a means of satisfactorily maintaining the viability and pathogenicity of *H. bovis*.

Only smooth colonies were noted on initial isolations of *Mor. bovis* from acute cases of bovine keratitis, and the colonies upon transfer remained smooth for approximately three to four months; then, intermediate, rough, and dwarf colonies appeared. This variation was not consistent, and reversion of the various types back to the smooth-type colonies occurred frequently. The rough and intermediate colonies had a smaller zone of *beta* hemolysis, measuring approximately ½ to 1½ mm. in width. The borders of some of the colonies took on a shelflike appearance, but this finding was not consistent in subculture. The dissociants rendered litmus milk slightly alkaline; no evidence of peptonization, as that which occurred in recently isolated cultures, was apparent. Baldwin[6] observed rough cultures on the original isolation from infective material, and he noted that attempts to maintain this variation failed and that the "R" colonies reverted to the "S" type. He cited a similar experience by Oag[47] who worked with *Moraxella lacunata* (bacillus of Morax-Axenfeld).

After a prolonged study of *Mor. bovis*, a survey was made of its incidence in acute cases of bovine keratitis in Kansas cattle. The organism was consistently present and was easily recovered from 92 of a total of 95 cases (96%). Jones and Little[33] reported on their observations of 24 cases of acute ophthalmia from which they isolated a characteristic diplobacillus in each case (100%). Baldwin[5] found *H. bovis* in 93 of 112 infected eyes (83%).

Nasal secretions were positive in 3 of 5 acute cases of infectious bovine keratitis. Jones and Little[34] found that the bacilli were eliminated from the nasal passage in their experimentally infected cases, and they were of the opinion that the phenomenon was associated with early infections. They did not recover the organism from the nasal passages of a large number of well-established cases and offered the explanation that "the irregularity of the elimination of the organism through the nostril may be explained by the effect of inflammation on the tear duct." Reid and Anigstein[51] found that the nasal exudate was as virulent for susceptible animals as the eye exudate.

Attempts were made to isolate the bacilli from the blood stream of infected cattle, but results were negative. Preliminary attempts were made to demonstrate circulatory antibody by means of a macroscopic agglutination test, but the limited amount of work performed revealed negative results. Baldwin[5] was not able to detect systemic antibodies either before or after inoculation in the serum of 11 experimental calves by precipitation or complement-fixation tests. Farley[23] prepared an antiserum from 3 calves that had recovered from acute keratitis. He gave 2 calves three injections each of the serum at weekly intervals, and two weeks after the third injection exposed the calves to virulent eye secretions. Both calves developed keratitis within ten days. Reid and Anigstein,[51] however, reported the demonstration of agglutinins specific for the diplobacillus in the serums of cattle convalescent from the natural or experimental infection.

As another phase of the incidence of Moraxella in this problem of pinkeye, a survey was made on normal cows and calves not affected with the disease and having a negative history for the condition (table 4). Normal sheep and lambs and those having an acute keratitis were also examined. A total of 11 calves, 25 cows, 12 lambs, and 70 sheep were negative in both eyes for evidence of the presence of *Mor. bovis*. Baldwin[5] was unable to recover *H. bovis* from the eyes of 20 normal cattle. It is realized that some apparently normal cattle may harbor *Mor. bovis* for a considerable time. Baldwin[5] demonstrated the presence of *H. bovis* in 1 animal for as long as 147 days after inoculation. Farley[24] stated that it has been proved experimentally that calves previously affected with the chronic type of infection were capable of transmitting the disease seven or eight months after apparent recovery.

An inflammatory eye condition typical of bovine keratitis (pinkeye) was produced experimentally in 4 isolated calves by the instillation of suspensions of *Mor. bovis* into the conjunctivas. This work verified the reports of Jones and Little[33, 34] and

Baldwin.[5] Allen[2] and Farley *et al.*[25] were not successful in their attempts to reproduce the eye condition by conjunctival instillations of the organism. The results of pathogenicity tests for *Mor. bovis* in rabbits and guinea pigs were negative and corroborate the research findings of Jones and Little,[33] Farley,[23] Farley *et al.*,[25] and Baldwin.[5] Sheep were not found to be susceptible to the infectious agent of bovine keratitis, which is in accord with the findings of Farley[23] and Baldwin.[5] However, Reid and Anigstein[51] reported 1 sheep and 2 goats to be susceptible to a hemolytic diplobacillus.

Reid and Anigstein[51] stained impression conjunctival smears with Giemsa's stain; some of the epithelial cells were found to contain small coccal and diplococcal bodies within the cytoplasm which closely resembled the RLO found in the conjunctival epithelium in ophthalmia of goats as reported by Coles,[14] and in sheep and cattle as reported by Donatien and Lestoquard.[22] Because foreign workers[12, 13, 18, 19, 20, 21, 35, 39, 42, 46, 48, 53, 60] have described an RLO as the etiological agent of infectious keratitis of cattle and/or sheep, it was thought expedient to study the eyes of normal and diseased cattle and sheep by means of conjunctival swabs and scrapings. *Moraxella bovis* was consistently isolated by means of conjunctival swabs from the cattle acutely affected with keratitis but was not isolated in a single instance from acutely affected sheep. Pleuropneumonia-like organisms or RLO were demonstrated in the conjunctival scrapings of 61 sheep affected with an acute keratitis, however. Chromatin granules were present in the conjunctival epithelial cells of normal and diseased cattle and sheep, often scattered among the cells as a result of ruptured pigmented cells. These granules of chromatin resembled somewhat the PPLO or RLO. An unknown type of organism was observed in the conjunctival cells of normal and diseased sheep but not in any of the cattle. Coles,[13, 14] Abdussalam,[1] and Shirlaw and Sankarnarayan[58] reported finding an unknown organism in the conjunctival scrapings of sheep and goats. Giemsa-stained slides made from conjunctival scrapings of sheep were sent to Dr. Albert B. Sabin* for his observation. He found microörganisms that were indistinguishable from the microörganisms of the pleuropneumonia group; the unknown organism did not represent any microörganism with which he was familiar.[56] Sabin[54] and Sabin and Johnson[55] reported the findings of pathogenic pleuropneumonia-like microörganisms in the conjunctiva and nasal mucosa of mice.

As in the case of other bacterial agents, failure to reproduce the disease in laboratory animals led many to question the etiological significance of *Mor. bovis*. However, keratitis of cattle in Kansas, based on the experimental work performed and the findings made in field investigations, is caused by *Mor. bovis*. Koch's postulates appear to be complete, and it may be reasonably concluded that *Mor. bovis* is the causal agent of keratitis of cattle (pinkeye). Pleuropneumonia-like organisms or RLO were not factors in the causation of infectious bovine keratitis.

SUMMARY AND CONCLUSIONS

1) *Moraxella bovis* was isolated from 92 of 95 cattle (96%) acutely affected with keratitis.

2) *Moraxella bovis* was not isolated from the eyes of 36 normal cattle having a negative history for keratitis.

3) An inflammatory eye condition typical of field cases of pinkeye was reproduced experimentally in 4 calves using a pure culture of *Mor. bovis*.

4) The incubation period of experimental keratitis in cattle caused by *Mor. bovis* varied from fifteen to twenty-one days.

5) Six cattle known to have had an acute attack of keratitis were not susceptible to reinoculation of *Mor. bovis* one year following experimental infection. This suggests the possibility of the development of an immunizing agent.

6) *Moraxella bovis* was not isolated from the eyes of 15 normal sheep and 67 sheep which showed evidence of an acute keratitis.

7) Guinea pigs, rabbits, and sheep were resistant to the inoculation of heavy suspensions of *Mor. bovis*.

8) *Moraxella bovis* was not isolated from the blood of cattle acutely affected with keratitis.

9) Rough, intermediate, and dwarf forms of *Mor. bovis* were recognized after prolonged subculturing.

10) The maintenance of cultures of *Mor.*

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bovis by desiccation rather than by frequent transfers is an effective method of preservation.

11) *Moraxella bovis* exhibited outstanding morphological, hemolytic, and proteolytic characteristics.

12) Cattle affected with keratitis caused by *Mor. bovis* may harbor the organism for one year or longer.

13) Pleuropneumonia-like microörganisms or rickettsial-like organisms were not demonstrated in the conjunctival scrapings of 36 normal cattle and 23 cattle which were affected with an acute keratitis.

14) An unknown organism was recognized in conjunctival scrapings of 4 normal sheep and in the eyes of 23 sheep affected with an acute keratitis.

15) Melanin granules were demonstrated in Giemsa-stained conjunctival scrapings of 28 of 36 normal cattle, 16 of 23 cattle affected with a keratitis, 12 of 15 normal sheep, and 58 of 67 sheep affected with a keratitis.

16) Pleuropneumonia-like microörganisms were demonstrated in the conjunctival scrapings of 55 sheep affected with an acute keratitis.

17) Keratitis of an infectious nature, as encountered in cattle in the state of Kansas, is caused by *Mor. bovis;* therefore, infectious bovine keratitis (pinkeye) would appear to be the appropriate nomenclature for the disease.

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Effect of Antibiotics in Diluted Bull Semen on Vibrio Fetus

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VIBRIO FETUS infection was found to be an important cause of temporary sterility, as well as abortion, by Plastridge *et al.*[1, 2, 3] Their work also suggested the possibility of bulls spreading infection, since the herd sires in several infected herds gave positive agglutination test reactions.

Recently, Stegenga and Terpstra, in three papers,[4, 5, 6] presented evidence that *V. fetus* is the usual cause of "Enzoötische steriliteit" of cattle in Holland, and that infection can be transmitted by mating healthy cows to infected bulls. They were able to isolate *V. fetus* from the semen of several suspected bulls.

Infection of heifers by artificial insemination with diluted semen containing added *V. fetus* cells was accomplished by Plastridge *et al.*[3] and Terpstra and Eisma.[7] The addition of antibiotics, particularly streptomycin and penicillin, to diluted bull semen to increase conception rates is a common practice of artificial breeding associations. Whether such treatment will render inactive *V. fetus* organisms that may be present is of interest.

Incomplete experiments of Roberts *et al.*[8] and Easterbrooks *et al.*[9] suggest that the addition to semen diluent of 500 units or μg. each of penicillin and streptomycin, or 500 μg. of streptomycin alone, will inactivate *V. fetus* organisms.

The present investigation was undertaken to determine the effect of various antibiotics in diluted bull semen on *V. fetus*.

EXPERIMENTAL

Semen Diluent.—A commercial semen diluent (Ortho) was used in the majority of the tests. However, a yolk-citrate diluent with or without added sulfanilamide (0.5%) gave results comparable with those obtained with the commercial diluent. Tests made with and without added semen were similar, hence semen was omitted after the early trials. It is also of interest that the *in vitro* tests conducted showed no evidence that the spermatozoa themselves were harmed as the result of adding *V. fetus* to the diluted semen.

Antibiotic-Diluent Mixtures.—Solutions of each antibiotic were prepared in sterile distilled water in concentrations such that the addition of from 0.02 to 0.2 ml. to 2.0 ml. of diluent yielded the desired final concentration. The mixtures were stored overnight at 5 C., inoculated the following forenoon, and either returned to the refrigerator or a 37 C. incubator, as indicated by the experiment.

Inoculum.—Two strains of *V. fetus* of bovine origin which grew well in thiol medium (Difco) were used. Tubes of thiol medium were inoculated with cells of stock cultures and incubated forty-eight hours. Each strain was used to inoculate two tubes of thiol medium and two tubes of thiol agar (thiol medium plus 0.005% glutathione and 2% agar).[3] After forty-eight hours' incubation, these cultures were used to prepare a suspension of cells having a density corresponding to about 5.0 on the MacFarland nephelometer scale. One-tenth-milliliter amounts were added to each tube of diluent-antibiotic mixture.

In tests to determine the bacteriostatic effect and stability of antibiotics in thiol medium kept at 37 C., a suspension with a density of about 1.5 on the MacFarland scale was used. A 4-mm. loopful of this suspension was inoculated into the test mediums.

Tests for Viable Cells.—The diluent-antibiotic-*V. fetus* mixtures were tested for viable cells at intervals of from one hour to four days. In the first experiments, a 4-mm. loopful was streaked on blood agar and the plates incubated in jars in which 10 per cent of the air was replaced by CO_2. The results obtained with blood agar were so erratic that tubes of thiol medium were inoculated with the test material and examined for growth after three, five, six, seven, and eight days' incubation. This procedure gave fairly consistent results in repeated trials. In questionable instances, resulting from turbidity caused by the diluent, transplants to fresh thiol medium were made.

RESULTS

Inactivation of Vibrio Fetus in Diluent Containing Antibiotics.—The results ob-

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From the Department of Animal Diseases, Storrs Agricultural Experiment Station, University of Connecticut, Storrs.

Aureomycin HCl with sodium glycinate was furnished by Lederle Laboratories Division, American Cyanamid Company, Inc., Pearl River, N. Y.; bacitracin by Commercial Solvents Corporation, Terre Haute, Ind.; gramicidin and tyrothricin by S. B. Pennick and Co., New York, N. Y.; penicillin sodium amorphous G and dihydrostreptomycin sulfate by Merck & Co., Inc., Rahway, N. J.; polymyxin D by Stamford Research Laboratories, Stamford, Conn.; and terramycin HCl by Chas. Pfizer & Co., Inc., Brooklyn, N. Y. A portion of the Ortho diluent was supplied by Ortho Research Foundation, Raritan, N. J.

TABLE 1—Semen Diluent Containing Vibrio Fetus and Antibiotics Stored at 5 C.

Antibiotic	Units of μg. per ml.	Thiol medium inoculated with diluent stored 1 hr.	1 day	2 days	4 days
Streptomycin	150	+	+	+	+
	200	+	+	+	+
	300	±	±	±	±
	500	—	—	—	—
Penicillin	2,000	(5)	(5)	(6)	(8)
	4,000	(7)	(5)	(6)	(8)
	6,000	(7)	(6)	(6)	—
	8,000	(7)	—	—	—
Aureomycin	500	+	+	+	(7)
	750	+	+	+	(7)
	1,000	+	(6)	(8)	—
Terramycin	500	+	+	+	(7)
	750	(7)	(7)	+	(7)
	1,000	(7)	(7)	(7)	(7)
Polymyxin	320	+	(6)	(6)	(6)
	640	+	(6)	(6)	(7)
	1,280	(7)	(7)	—	—
Bacitracin Gramicidin Tyrothricin	16,000	+	+	+	+

+ = growth in three days; — = no growth in eight days; ± = variable results; (No.) = days required for growth when no growth was visible in three days.

tained when diluent-antibiotic-*V. fetus* mixtures stored at 5 C. were inoculated directly into thiol medium are summarized in table 1.

Streptomycin in concentrations of 500 μg. per milliliter of diluent prevented the growth of *V. fetus*. Variable results were obtained at the 300-μg. level, and growth was consistently obtained from the mixtures containing 200 μg. or less. It is of interest that results obtained after four days' storage at 5 C. were no different than those obtained one hour after the addition of *V. fetus*. As will be shown later, the negative results obtained at the 500-μg. level were due to the bacteriostatic action of streptomycin carried over with the inoculum and the stability of streptomycin in thiol medium at 37 C.

Penicillin gave somewhat different results from those obtained with streptomycin. Growth occurred in thiol tubes inoculated with mixtures containing up to 6,000 units per milliliter, but only after prolonged incubation. This indicates that growth occurred after the penicillin carried over in the inoculum had disintegrated. At the 8,000-unit level, the *V. fetus* cells were apparently destroyed within twenty-four hours.

Aureomycin, terramycin, and polymyxin gave results similar to those obtained with penicillin but at lower concentrations.

Bacitracin, gramicidin, and tyrothricin were relatively inert against *V. fetus* under the conditions of the experiment. Growth was obtained in three days from mixtures containing 16,000 μg. per milliliter of any one of these antibiotics.

Bactericidal Action of Streptomycin Against Vibrio Fetus.—The preceding experiments indicate that, with the possible exception of streptomycin, none of the antibiotics tested could be used in semen diluent in concentrations sufficient to render inert any *V. fetus* organisms that might be present without impairing the fertility of sperm cells.

The level of streptomycin required to kill *V. fetus* in diluent at 5 C. and at 37 C. was determined. Duplicate sets of centrifuge tubes were prepared containing 2 ml. of diluent to which from 125 to 2,000 μg. of streptomycin per milliliter were added. The mixtures were inoculated in the usual way and one set stored at 5 C. and one at 37 C. The latter were placed in a water bath and brought to a temperature of 30 to 35 C. before inoculation. At intervals, tubes were removed from each set and the cells thrown down by centrifugation. The cells were washed once with sterile saline and then resuspended in 2 ml. of sterile saline. A 4-mm. loopful of each suspension was inoculated into a tube of thiol medium. The results are given in table 2.

At 5 C., *V. fetus* survived three days in diluent that contained 1,000 μg. of streptomycin. Subcultures made in thiol medium before centrifuging yielded no growth during eight days of incubation, indicating that before centrifugation sufficient streptomycin was carried over with the inoculum

TABLE 2—Bactericidal Action of Streptomycin in Semen Diluent Against Vibrio Fetus*

Storage temperature	Micrograms of streptomycin per ml.	Viable cells after 1 hour	1 day	3 days
5 C.	1,000	+	+	+
	1,500	±	+	±
	2,000	—	—	—
37 C.	125	+	+	—
	250	+	+	—
	500	+	—	—
	750	+	—	—
	1,000	±	—	—

*Diluent with streptomycin and *Vibrio fetus* was centrifuged, the sediment suspended, the suspension recentrifuged, the sediment resuspended in sterile saline, and one 4-mm. loopful inoculated into fresh thiol medium.

+ = growth in three days; — = no growth in eight days; ± = variable results.

to prevent growth. Variable results were obtained at the 1,500-μg. per milliliter level, however, no viable cells were recovered from diluent that contained 2,000 μg. per milliliter.

At 37 C., variable results were obtained after one hour with diluent that contained 1,000 μg. per milliliter. However, 500 μg. per milliliter of diluent destroyed *V. fetus* in less than twenty-four hours, and no viable cells were found in diluent that contained 125 μg. after three days' storage at 37 C.

Stability of Antibiotics in Thiol Medium at 37 C.—The results given in table 1 suggested that, with the exception of streptomycin, the antibiotics used deteriorated at 37 C. To obtain further information, different concentrations of streptomycin, penicillin, and aureomycin were prepared in tubes of thiol medium. These were stored at 37 C. and inoculated at intervals with *V. fetus* cells. The results are given in table 3.

Streptomycin in a concentration of 1.0 μg. per milliliter of diluent was sufficient to prevent the growth of *V. fetus*. No evidence of deterioration occurred during the three days' storage at 37 C. Since no visible growth occurred on prolonged incubation, it seems reasonable to assume that the streptomycin remained active for at least eight days.

Penicillin deteriorated rapidly. At levels of 1,000 to 3,000 units per milliliter, growth was obtained after six days' incubation in tubes inoculated one hour after the penicillin was added to the medium. Replicate sets of tubes inoculated after storage for one day showed little or no evidence of growth inhibition. Thiol medium that contained 4,000 units of penicillin per milliliter and that was inoculated one hour after addition of penicillin to the medium failed to show growth. However, similar tubes inoculated after storage at 37 C. for one day or longer showed growth in less than four days. In other tests, as little as four to eight units of penicillin per milliliter prevented visible growth in three days.

Aureomycin in thiol medium also deteriorated at 37 C. but somewhat less rapidly than did penicillin. From 1 to 2 μg. per milliliter inhibited visible growth for three days. However, medium that contained up to 500 μg. per milliliter and that was inoculated one hour after the addition of aureomycin yielded growth after eight days' incubation. Similar tubes inoculated after two days' storage showed no evidence of growth inhibition. In thiol medium that contained either 750 or 1,000 μg. per milliliter and that was inoculated one hour after addition of the antibiotic, no growth was obtained during the eight-day period of incubation. This suggests that the *V. fetus* cells were destroyed at these levels, since similar tubes stored at 37 C. for three days showed no evidence of growth inhibition. If the cells had survived, growth should have appeared by the end of the incubation period.

TABLE 3—Stability of Antibiotics in Thiol Medium at 37 C.

Antibiotic	Units or μg. per ml.	Medium stored at 37 C. and inoculated after 1 hr.	1 day	2 days	3 days
Streptomycin	0	+	+	+	+
	0.5	+	+	+	+
	1.0	—	—	—	—
Penicillin	1,000	(6)	+	+	+
	2,000	(6)	+	+	+
	3,000	(6)	(4)	+	+
	4,000	—	(4)	+	+
Aureomycin	250	(8)	(5)	+	+
	500	(8)	(5)	+	+
	750	—	(5)	(5)	+
	1,000	—	(5)	(5)	+

+ = growth in three days; — = no growth in eight days; ± = variable results; (No.) = days required for growth when no growth was visible in three days.

SUMMARY AND CONCLUSIONS

Direct subculturing of semen diluent-antibiotic-*Vibrio fetus* mixtures, stored at 5 C., into thiol medium showed that: (1) 500 μg. of streptomycin per milliliter of diluent prevented growth of the organism; (2) up to 6,000 units of penicillin per milliliter failed to prevent growth if the inoculated mediums were incubated from six to eight days; (3) 1,000 μg. per milliliter of either aureomycin or terramycin and 640 μg. of polymyxin per milliliter failed to prevent development of *V. fetus* after prolonged incubation; and (4) bacitracin, gramicidin, and tyrothricin, at the 16,000-μg. per milliliter level, showed no effect on *V. fetus*.

The minimum concentration of streptomycin required to destroy *V. fetus* in less than twenty-four hours in diluent, stored at 5 C., was 2,000 μg. and, at 37 C., 500 μg. per milliliter. The minimum concentration to inhibit growth was 1.0 μg. per milliliter.

Streptomycin was found to be remark-

ably stable in thiol medium stored at 37 C. for eight days. Under similar conditions, penicillin, aureomycin, terramycin, and polymyxin underwent rapid deterioration.

The results obtained showed that the addition of 500 μg. of streptomycin per milliliter of diluted bull semen would not destroy *V. fetus* during storage at 5 C. The results also indicated that *V. fetus*, if present, in diluted bull semen treated with 500 μg. of streptomycin per milliliter would be rendered incapable of producing infection at the time of or following insemination, owing to the stability and increased activity of streptomycin at body temperature.

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Corrigenda

On pages 14 and 18 of the January issue of the *American Journal of Veterinary Research*, "lethal dose$_{50}$" was erroneously used instead of "infective dose$_{50}$". Both of these errors appear in tables 1 of the two articles on foot-and-mouth disease virus by H. L. Bachrach, Ph.D.

Preparation of an Improved Antigen for Anaplasmosis Complement-Fixation Tests

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A METHOD FOR the production of antigen for anaplasmosis complement-fixation tests was outlined in 1949 by Mott and Gates[1] of the Bureau of Animal Industry (BAI), United States Department of Agriculture. The antigen was prepared from the parasitized erythrocytes of splenectomized calves in the acute stage of anaplasmosis. The erythrocytes were collected, washed, and then lysed in carbon dioxide-saturated distilled water. Upon overnight refrigeration at 4 to 6 C., a precipitate was formed. The washed precipitate was resuspended in distilled water, the pH adjusted to 7.0, and lyophilized. Frequently, antigens prepared by this method were found to be unsatisfactory because of their anticomplementary nature and/or their nonspecific activity.

PROCEDURE

It was felt that if the antigen could be obtained in a more concentrated form, perhaps the activity of the interfering properties could be reduced. We utilized high speed centrifugation techniques as a means of concentrating the antigen. All blood used in the study was obtained from splenectomized calves during the acute stage of anaplasmosis. These calves were inoculated with *Anaplasma marginale* (Louisiana strain 3272) which was obtained from Dr. D. W. Gates of the BAI. The blood was drawn from the animals at that time when the number of parasites per cubic millimeter reached a maximum as determined by this equation: number of red cells/cmm. by fraction of red cells parasitized = number of parasites/cmm.

All blood was collected into sterile 2-liter flasks containing 50 ml. of 20 per cent sodium citrate solution. The plasma was removed and the cells washed four times in physiological saline by centrifugation at 2,500 revolutions per minute (r.p.m.) for twenty minutes. The packed cells were lysed by adding distilled water in the proportion of 15 volumes of distilled water to one volume of packed cells and allowed to stand for ninety minutes at 4 to 6 C. This material was centrifuged in Servall angle centrifuges, using nitrocellulose tubes, for thirty minutes at not less than 5,000 r.p.m. The sediments were pooled into several tubes and washed repeatedly in distilled water until the supernatant was hemoglobin-free. The sediment was triturated with a glass stirring rod and resuspended in distilled water. Following another centrifugation, two different, though poorly defined layers, could be observed. The bottom one appeared as a heavy, well-packed layer, grayish in color and the upper layer consisted of a light, fluffy, brownish mass. This upper layer was partially removed with an aspirating pipette and the sediment again ground, resuspended, and centrifuged in order to remove more of the fluffy material. The gradual removal of the upper layer was necessary to avoid too great a loss of the packed lower layer. This process was repeated until a smooth homogeneous button-like sediment remained. The sediment was washed free from the centrifuge tubes with a small quantity of distilled water, pooled, and distributed into 20-ml. vaccine bottles in 7-ml. quantities. The bottles were sealed with rubber stoppers, shell-frozen, and lyophilized for eighteen hours. The dried antigen was then weighed and resuspended in the proportion of 100 mg. of antigen to 20 ml. of veronal bicarbonate-buffered salt solution, pH 7.3 to 7.4. The resuspended antigen was finally dispensed into ampules in 1-ml. amounts and lyophilized for eighteen hours.

Reagents of the hemolytic system were standardized spectrophotometrically, and were employed in a complement-fixation procedure developed by Kent.[2] The total volume of reagents was 1.5 ml., 0.3-ml. aliquots being reserved for the five essential reagents of the complement-fixation procedure. Preliminary incubation was for one hour in the water bath at 37 C. A 2 per cent cell suspension sensitized with an optimal concentration of hemolysin was then added in 0.6-ml. quantities, and thirty minutes in the water bath at 37 C. was allowed for hemoylsis. Percentage of hemolysis in the complement-fixation tests was read by comparison with prepared standards.

Table 1 compares the results obtained from the titration of antigens prepared by the previously described method (A) and those obtained from the titration of antigens prepared

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We are deeply indebted to Dr. D. W. Gates of the Bureau of Animal Industry, United States Department of Agriculture and Dr. W. M. Mohler of the Pathology Division of the United States Department of Agriculture for supplying stock positive serum and the inoculum used in these studies.

from the same lots of blood by the method of Mott and Gates (B). The two antigens prepared from the blood of calf A 3235 were comparable in antigenic activity, but exhibited distinct differences in anticomplementary properties. Thus, the antigen prepared by method B was considered to be completely unsatisfactory because of anticomplementary activity; while the antigen prepared by high speed centrifugation showed only slight inhibition of hemolysis, and this at a dilution considerably lower than would be optimally employed in complement-fixation tests.

In the case of calf 1, antigen prepared by method B showed relatively little antigenic activity, whereas that prepared by method A had a satisfactory antigenic titer. Neither of the antigens displayed any anticomplementary or nonspecific activity.

Calf 2 was inoculated with 250 ml. of packed cells which were obtained when calf 1 was exsanguinated. When this animal failed to show any symptoms of anaplasmosis, calf 3 was inoculated with cells from the same carrier animal that furnished inoculum for calf 1.

The antigen prepared by method A from the blood of calf 3 was comparable in antigenic activity to that obtained by the same method from the blood of calf 1. This antigen gave a much more satisfactory range between antigenic and anticomplementary activity than did the antigen processed by method B.

The 250 ml. of packed cells collected from calf 3 during the acute stage of anaplasmosis served as inoculum for calf 4. High titered antigens were obtained and, as in the previous cases, the anticomplementary activity was considerably reduced by concentration methods.

DISCUSSION

All four antigens prepared in this study by high speed centrifugation techniques were found to be superior to those prepared by the method of Mott and Gates.[1] In three antigens, the superiority resulted from decreased anticomplementary activity. This reduction produced a much greater range between antigenic and anticomplementary activities.

In the case of the remaining antigen, concentration techniques proved to be valuable. The antigen prepared by high speed centrifugation had an antigenic titer of sufficient magnitude to be successfully employed in complement-fixation procedures, while that prepared by the method of Mott

TABLE 1—Comparison of Results Obtained from Titrations of Antigens Prepared by Methods A and B

Calf. No.		A								B							
		Serum dilutions						Antigen controls		Serum dilutions						Antigen controls	
		1/6	1/9	1/12	1/18	1/24	1/36	+ NS*	+ Saline	1/6	1/9	1/12	1/18	1/24	1/36	+ NS	+ Saline
A 3235	1/3	0	0	0	5	15	30	95	95	0	0	0	0	5	20	10	15
	1/6	0	0	0	0	0	5	100	100	0	0	0	0	0	15	70	50
Antigen	1/9	0	0	0	0	0	15	100	100	0	0	0	0	0	20	100	70
dilutions	1/12	0	0	0	0	5	20	100	100	0	0	0	10	20	40	100	95
	1/18	0	0	10	30	40	55	100	100	0	5	20	55	80	90	100	100
	1/24	5	30	40	50	70	90	100	100	70	90	100	100	100	100	100	100
Serum	+	100	100	100	100	100	100	...	...	100	100	100	100	100	100	...	...
controls	—	—	—	—	—	—	—	100	...	—	—	—	—	—	—	100	...
1	1/3	0	0	0	0	5	30	100	100	20	35	55	75	80	90	100	100
	1/6	0	0	0	0	5	20	100	100	80	95	100	100	100	100	100	100
Antigen	1/9	0	0	0	5	30	40	100	100	100	100	100	100	100	100	100	100
dilutions	1/12	0	0	20	50	80	90	100	100	100	100	100	100	100	100	100	100
	1/18	5	35	85	95	100	100	100	100	100	100	100	100	100	100	100	100
	1/24	90	95	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Serum	+	100	100	100	100	100	100	...	...	100	100	100	100	100	100	...	...
controls	—	—	—	—	—	—	—	100	...	—	—	—	—	—	—	100	...
3	1/3	0	0	0	0	0	10	90	100	0	0	0	0	15	25	0	10
	1/6	0	0	0	0	5	10	95	100	0	0	0	0	0	5	40	60
Antigen	1/9	0	0	0	5	10	25	100	100	0	0	0	5	20	25	70	80
dilutions	1/12	0	0	10	20	40	50	100	100	0	0	10	15	25	45	85	100
	1/18	60	80	85	90	90	100	100	100	5	10	15	25	30	60	100	100
	1/24	100	100	100	100	100	100	100	100	10	20	35	60	80	90	100	100
Serum	+	100	100	100	100	100	100	...	...	00	100	100	100	100	100	...	...
controls	—	—	—	—	—	—	—	100	...	—	—	—	—	—	—	100	...
4	1/3	0	0	0	10	10	20	90	100	0	0	0	0	10	15	0	0
	1/6	0	0	0	0	0	0	100	100	0	0	0	0	0	5	60	80
Antigen	1/9	0	0	0	0	0	5	100	100	0	0	0	0	10	20	90	100
dilutions	1/12	0	0	0	5	10	15	100	100	0	0	0	10	10	30	95	100
	1/18	0	0	0	5	20	25	100	100	0	0	5	15	25	45	100	100
	1/24	0	0	10	10	30	50	100	100	5	10	30	60	95	100	100	100
Serum	+	100	100	100	100	100	100	...	...	00	100	100	100	100	100	...	...
controls	—	—	—	—	—	—	—	100	...	—	—	—	—	—	—	100	...

0 = complete fixation; 100 = no fixation; *NS = normal bovine serum (1/10).

and Gates had very little antigenic activity. It should be noted that the blood in question showed a low degree of parasitization, and it is believed that failure to concentrate the relatively few parasites accounted for the poor antigen.

It was of considerable interest that antigens prepared from parasites obtained during the first passage from the carrier state showed somewhat lower antigenic activity than those prepared from parasites which had been passed through 2 or more splenectomized calves.

The quantity of antigen obtained by high speed centrifugation methods compared favorably with that obtained by the method of Mott and Gates. We believe that a more satisfactory method for the preparation of antigen for anaplasmosis complement-fixation tests has been established.

SUMMARY

1) Four splenectomized calves were inoculated with *Anaplasma marginale* (Louisiana strain 3272) in order to prepare complement-fixation antigens.

2) Antigens were prepared by the method of Mott and Gates and also by a method in which high speed centrifugation techniques were utilized.

3) Titration results showed that antigens produced by high speed centrifugation methods were superior. This superiority was because of the removal of a greater amount of extraneous material which resulted in an increased concentration of parasites and a decreased anticomplementary activity.

4) Comparable amounts of antigen were obtained by the high speed centrifugation method.

5) Antigens prepared from parasites which had gone through two or more animal passages gave higher titers.

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Studies on the Experimental Treatment of Bovine Brucellosis

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WITH THE DISCOVERY of each disease, a satisfactory method of therapy is always sought. A satisfactory and practical treatment for brucellosis in cattle remains to be developed. It is realized that even though an effective therapy is developed it probably would not be the answer to the control and eradication of bovine *Brucella abortus* infection. Effective bovine brucellosis therapy can only be an aid in the over-all control and eradication program.

Recent advances in the treatment of human brucellosis have stimulated new interest in the bovine infection in the field of veterinary medicine. In addition, the development of the sulfonamides and the various antibiotics has generated new impetus to the difficult problem of brucellosis therapy in cattle.

Brucellosis therapy in cattle is beset with numerous problems. Initially a chemical, drug, biological agent, or antibiotic must, either alone or in combination, be nontoxic to the host at the effective therapeutic dosage. It is essential that a satisfactory margin of safety exist between the therapeutic and toxic dosage level. Secondly, after an effective therapeutic agent is found, it is necessary that some practical method be developed for determining when a treated animal is completely free of the infective agent. Thirdly *Br. abortus* infection in the bovine animal exists as a more or less chronic infection with localized tissue areas harboring the organisms, and not as an acute bacteriemia as is thought to be the case in the infected human patient. Usually, acute infections respond more readily to therapy than do chronic infections.

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LITERATURE

Prior to the advent of the sulfonamides, numerous chemical agents were used in an attempt to alter the Brucella-agglutinin titer of reacting cattle. Graham *et al.*[1] observed that acriflavine, trypan blue, trypacrin A, colloidal carbon, thionine, alkali, hypoiodite, and pyronine failed to affect the blood-serum agglutinin titer. Similar observations were made by Gotze and Muller,[2] who concluded that no known drug at the time of their researches had any definite value in bovine brucellosis therapy. Numerous chemical agents were employed by Cordier and Menanger[3] to treat bovine brucellosis, but none was found that would affect the blood-serum agglutination titer or enhance the resistance of guinea pigs to Brucella infection from milk obtained from mammary quarters shedding organisms. Cotton and Swope[4, 5] have recently shown that sodium para-amino-benzoic acid, administered subcutaneously every four hours for twenty-one days at a dosage level of 3 Gm. per kilogram of body weight, would produce complete tissue sterilization provided the treatment was initiated three days after the guinea pigs were infected with Brucella. These workers found that when therapy was started fourteen days after the guinea pigs were infected, only 80 per cent of the infected individuals showed tissue sterilization.

Sulfonamides have been employed during the past decade in an attempt to treat Brucella infection. Huddleson[6] was the first to report that the sulfonamides were valueless in brucellosis therapy in cattle and guinea pigs. Chinn[7] treated infected guinea pigs at the time of infection with sulfanilamide, and observed sterile spleens and livers from the autopsied pigs. Hamman and Huddleson[8] showed that protosil and sulfanilamide had little effect on the blood-serum agglutination titer or the shedding of Brucella organisms in the milk from Brucella-infected cows treated over a period of five to seven weeks. Rajcevic and Okljesa[9] administered streptozol (sulfanilamide) for ten-day intervals over a period of six months to cows that were naturally infected with *Br. abortus* and observed that little effect was obtained on the blood-serum agglutinin titer. It was also observed that streptozol did not prevent nonreacting cows from becoming reactors following contact exposure to infected animals. Dumaresq[10] employed massive doses

of sulfanilamide in the treatment of guinea pigs and showed that the drug retarded the Brucella infection. This worker also treated 2 naturally occurring cases of bovine brucellosis with 30 to 60 Gm. of sulfanilamide daily for eleven to thirteen days, and observed that the dosage level was toxic for the host, but did not cause the blood-serum agglutinin titer to recede nor did it free the mammary gland of organisms.

Hamman and Huddleson[11] observed that sulfapyridine had some bacteriostatic effect on Brucella *in vitro*, but did not alter the agglutinin titers nor the organisms in infected guinea pigs. Wilson and Maier[12] treated Brucella-infected guinea pigs at the time of infection with sulfapyridine, and observed that the livers and spleens were sterile at the time of autopsy. Live, Stubbs, and Gardiner[13] obtained only moderate results in treating Brucella-infected cows known to shed organisms in the milk with sulfapyridine. These investigators[14] also employed sulfathiazole in the treatment of cows known to eliminate *Br. abortus* in the milk, obtaining only mediocre results.

Schuhardt, Rich, and Beal[15] treated cattle with sulfadiazine for Brucella infection, but reported no cures. Huddleson[16] fed sulfadiazine at the rate of 0.2 Gm. daily per guinea pig for thirty days. The guinea pigs were experimentally infected with either *Brucella suis* or *Brucella melitensis*. At the close of the first 24-hour feeding period, each pig was injected intraperitoneally with 2 ml. of normal rabbit serum. The results were encouraging and indicated that experimentally induced Brucella infection in guinea pigs might be cured rapidly.

With the discovery of penicillin, there developed a few experiments designed to treat brucellosis. T'ung[17] found that eight of 15 strains of Brucella tested showed some susceptibility to penicillin *in vitro*. Berman, Irwin, and Beach[18] found that penicillin affected no cures in Brucella-infected cattle. Bunnell, Hutchings, and Donham[19] treated guinea pigs experimentally infected with *Br. suis* and found that penicillin in varying dosage levels, at various periods of time following exposure, failed to alter significantly the course of the disease.

Following the development of streptomycin, it was found that Brucella organisms are susceptible to its action *in vitro*.[20, 21, 22] Live, Sperling, and Stubbs[23] concluded that streptomycin-treated guinea pigs infected with *Br. abortus* were protected by the antibiotic. Gilman and LeGrow[24] treated guinea pigs infected with *Br. abortus* with streptomycin administered intraperitoneally for a short period of time and observed that, under the conditions of the experiment, the disease was not overcome nor altered in its course. Kelly and Henley[25] have shown that streptomycin is of little value in the treatment of guinea pigs infected with *Br. suis*. Similar results were obtained in the treatment of human patients with streptomycin alone.[26, 27]

Recently Kolmer[28] has reviewed the subject of the synergistic or additive activity of chemotherapeutic compounds against various microörganisms. This report has stimulated the combination of two or more drugs, antibiotics, or biological products in the treatment of infectious diseases.

Watts, Boley, and Greig[29] repeatedly treated 4 Brucella-infected cattle with sodium sulfamethazine and transfusions of whole blood or normal serum. The treatment did not reduce the blood-serum agglutinin titer nor did it prevent 3 of the cows from shedding Brucella in their milk.

In human medicine, Pulaski and Amspacher[27, 30] were the first to report that a combination of streptomycin and sulfadiazine would serve as successful therapy for human brucellosis. Other workers have confirmed this work and have shown that sulfadiazine synergizes with the activity of streptomycin in its action on Brucella elimination from the infected individual. As a result, a high proportion of acute human cases of brucellosis have been reportedly cured.[31-36]

Holm and McNutt[37] produced bacteriological cures in experimental brucellosis in guinea pigs with streptomycin and sulfadiazine administered subcutaneously and orally. The drug-antibiotic combination was as effective when administered ten days after the animals were infected as when treatment was initiated on the first day following infection. It was also found that the combination was as effective administered once daily as when given five times daily. Holm and Moore[38] observed that subcutaneous administration of streptomycin, plus oral administration of sulfadiazine on alternate days, was as effective as daily treatments to guinea pigs experimentally infected with *Br. abortus*. These workers also found that treatment every other day was as effective when initiated at the time of guinea pig infection as when it was started ten days following guinea pig infection. Under the conditions of the experiment, *Br. abortus* was not isolated from the spleens of 70 to 100 per cent of the treated guinea pigs.

Since the development of aureomycin, several reports have been published on its activity against Brucella infection. Spink *et al.*[39] administered aureomycin orally to human patients infected with *Br. melitensis*. The most recent information published on this experiment indicates that only three of the twenty-four treated patients developed a recurrence of fever and positive blood cultures.

Heilman[41] treated experimental Brucella infections in mice with aureomycin given orally and dihydrostreptomycin. He found the combination superior in effect to that of sulfadiazine-streptomycin and sulfadiazine-aureo-

TABLE 1—Effect of Sulfadiazine and Blood Plasma Transfusions on Brucellosis in Dairy Cattle

Cow No.	Pretreatment		Treatment	Post-treatment			
				12 days after treatment		16 days after treatment	
	Ave. blood titer	Milk culture		Ave. blood titer	Milk culture	Ave. blood titer	Milk culture
1	I 25,600	+	a. Plasma b. Sulfadiazine	I 12,800	—	I 6,400	—
5	25,600	+	a. Plasma b. Sulfadiazine	I 12,800	+	I 12,800	+
7	I 25,600	+	a. Plasma b. Sulfadiazine	I 12,800	+	I 6,400	+
9	I 12,800	+	a. Plasma b. Sulfadiazine	I 12,800	+	I 3,200	+
10	I 200	+	a. Sulfadiazine	I 200	—	I 400	—

I = incomplete agglutination; + = *Br. abortus* isolated from milk; — = unable to isolate *Br. abortus* from milk.

TABLE 2—Effect of Sulfadiazine and Colloidal Manganese on Brucellosis in Dairy Cattle

Cow No.	Pretreatment		Treatment	15 days after treatment	
	Ave. blood titer	Milk culture		Ave. blood titer	Milk culture
1	I 400	+	a. Colloidal manganese	I 800	+
4	12,800	+	a. Manganese b. Sulfadiazine	I 12,800	+
8	I 12,800	+	a. Manganese b. Sulfadiazine	I 12,800	+
10	I 6,400	+	a. Manganese	25,600	+

I = incomplete agglutination; + = *Br. abortus* isolated from milk; — = unable to isolate *Br. abortus* from milk.

TABLE 3—Effect of Sulfadiazine and Streptomycin on Brucellosis in Dairy Cattle

Cow No.	Pretreatment		Treatment	Post-treatment			
				12 days after treatment		30 days after treatment	
	Ave. blood titer	Milk culture		Ave. blood titer	Milk culture	Ave. blood titer	Milk culture
49	I 51,200	+	a. Streptomycin b. Sulfadiazine	I 25,600	—	I 25,600	+
361	51,200	+	a. Streptomycin intramuscularly b. Streptomycin intramammary c. Sulfadiazine	I 51,200	—	I 51,200	+
4	I 25,600	+	a. Streptomycin	I 25,600	+	I 12,800	+

I = incomplete agglutination; + = *Br. abortus* isolated from milk; — = unable to isolate *Br. abortus* from milk.

TABLE 4—Effect of Aureomycin and Streptomycin on Brucellosis in Dairy Cattle

Cow No.	Pretreatment		Treatment	30 days after treatment	
	Ave. blood titer	Milk culture		Ave. blood titer	Milk culture
1	I 3,200	+	a. Aureomycin* b. Streptomycin	I 3,200	+
11	12,800	+	a. Aureomycin b. Streptomycin	I 12,800	+
20	I 6,400	+	a. Aureomycin intramammary infusion	I 12,800	+
537	I 6,400	+	a. Aureomycin b. Streptomycin	I 6,400	+

I = incomplete agglutination; + = *Br. abortus* isolated from milk; — = unable to isolate *Br. abortus* from milk.
*Aureomycin discontinued at end of third day.

mycin combinations. Under the conditions of the experiment, it was found that no sulfa-antibiotic combination completely sterilized the mice spleens.

Herrell[48] treated three human cases of *Br. abortus* infection and one case of *Br. suis* infection with oral aureomycin and dihydrostreptomycin. No recurrence of fever or positive blood cultures were reported.

Holm and Moore [49] recently reported on the use of aureomycin and sulfadiazine administered subcutaneously and orally to guinea pigs experimentally infected with *Br. abortus*. The combination reduced the spleen size in the guinea pigs, but was not as effective as streptomycin and sulfadiazine in eliminating the infection. Best results were obtained when the treatment was initiated ten days following infection. The aureomycin alone and aureomycin plus sulfadiazine produced toxic effects in the guinea pigs.

Larsen and Gilman[42] treated 4 acutely affected cows with aureomycin. The aureomycin failed to arrest the Brucella infection, or to modify its usual course as observed in the treated cases.

Materials and Methods

The present study was undertaken in an attempt to determine the value of (a) sodium sulfadiazine administered intravenously and orally, accompanied by bovine blood plasma transfusions, accompanied by colloidal manganese intravenously; (b) sodium sulfadiazine administered intravenously and orally, accompanied by calcium chloride complex streptomycin intramuscularly; and (c) aureomycin administered intravenously, accompanied by calcium chloride complex streptomycin intravenously. The treatments were administered to mature cattle experimentally infected with *Br. abortus* BAI strain No. 2308, and to animals naturally infected. The presence of the disease in the artificially and naturally infected cows both before and following treatments was based upon actual isolation of the organism from the milk, vaginal secretions, or placenta, and positive blood-serum agglutination.

The infected cows described in groups 1, 2, and 3 were selected from two naturally infected private herds. Brucella infection had been present in these herds for slightly more than one year. Animals described in group 4 were selected from an artificially infected group with *Br. abortus* BAI strain No. 2308. Milk from these 20 agglutinin-positive animals was cultured, and 16 were selected on the basis of their consistent shedding of Brucella organisms.

The various groups were treated as follows:

Group 1 (table 1).—Cows 1, 5, 7, and 9 received 1,000 ml. of bovine plasma intravenously to which had been added 10 Gm. of sodium sulfadiazine. All animals in this group were given sulfadiazine orally every twelve hours for four days at the rate of approximately 1 gr. per pound of body weight per day. Bovine plasma was prepared from blood of animals negative to the Brucella agglutination test.

Group 2 (table 2).—Colloidal manganese was administered intravenously to each of 4 animals at the rate of 125 ml. per day for ten days. Animals 4 and 8 received in addition to the manganese, sulfadiazine orally by balling gun, at the rate of approximately 0.5 gr. per pound of body weight every twelve hours for eight days.

Group 3 (table 3).—Streptomycin was administered intramuscularly to each of the 3 animals in this group. The dosage rate was 5 mg. per pound of body weight every six hours for seventy-two hours. Animal 361 also received intramammary infusions of 0.5 Gm. of streptomycin per quarter per day for three days. Animals 49 and 361 received, in addition to streptomycin, sulfadiazine orally at the rate of 0.5 gr. per pound of body weight every twelve hours for four days.

Group 4 (table 4).—Aureomycin was administered intravenously to each animal of this group. The dosage was 5 mg. per pound of body weight per day, and administered every twelve hours. The treatment was continued for five days in all animals except cow 1, which developed a very severe phlebitis, and treatment was discontinued the third day. In addition to the above treatment, cow 20 received intramammary infusions of 200 mg. of aureomycin per quarter per day for three days. Cows 11 and 537 received, in addition to aureomycin, streptomycin intramuscularly at the rate of 5 mg. per pound of body weight per day for six days. Streptomycin was given every twelve hours.

Blood-serum agglutination tests and milk cultures were made before, during, and following treatment at frequent intervals throughout the experiments. In addition to the above observations, free sulfonamide, streptomycin, and aureomycin blood levels were determined at frequent intervals.

The results of the experiments are shown in tables 1, 2, 3, and 4.

In all cases, except for 2 animals which received colloidal manganese alone, there was an apparent cessation of the shedding of Brucella organisms from approximately the third day of treatment to three to ten days following treatment.

In all treated animals, except cow 1 and cow 10 in group 1, recurrent infection was noted. The necessity of disposal of all animals in group 1, sixteen days after treatment, did not permit further examination of the 2 nonshedders. However, it would appear probable that, had examinations been made at later

periods, organisms would have been recovered from these 2 animals also.

Only slight variation in the blood-serum agglutinin titers was noted when pretreatment and post-treatment titers were compared. The titers were about the same as in nontreated infected animals.

Free sulfadiazine in cattle to which the drug had been administered by balling gun varied from 1.0 to 3.0 mg. per 100 ml. in milk and ranged from 2.0 to 9.3 mg. per 100 ml. of blood serum. Blood levels of streptomycin in cattle treated intramuscularly varied from 12 to 66 units per milliliter, and aureomycin levels ranged from 0.2 mg. to 1.6 mg. per milliliter.

DISCUSSION

Sulfadiazine, streptomycin, aureomycin, and various combinations of these therapeutic agents caused a temporary cessation of shedding of Brucella organisms in the milk of treated animals. However, recurrent shedding occurred in 14 of 16 animals treated. From the 2 animals in which infection had no recurrence, milk and postmortem specimens were only available for sixteen days of observation.

The data seem to pose the question of just where the bacteriostatic effect of the drugs used takes place. It still remains to be established whether the bacteriostatic effect of the drug takes place *in vivo* or *in vitro*.

SUMMARY

1) Blood plasma, sulfadiazine, streptomycin, colloidal manganese, and aureomycin, singly or in combination, were administered to 16 cattle shedding Brucella organisms in their milk.

2) The use of the therapeutic agents, singly or in combination, apparently caused a temporary cessation in the shedding of Brucella organisms in most of the animals treated. Recurrent infection was noted in all but 2 animals in which studies were completed; however, the latter were under observation for only a sixteen-day period.

3) The use of therapeutic agents, singly or in combination, had no apparent affect on the blood agglutination titers of the animals treated.

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Porcine Leptospirosis

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LEPTOSPIRA POMONA was originally isolated in 1937 from a Queensland dairy farmer by Clayton, Derrick and Cilento.[1] Isolation of this organism from swine was first reported in 1942 by Johnson[2] in Australia. Subsequently, Gsell[3] incriminated *L. pomona* as the etiological agent of "swineherd's disease" and described the infection in man and in swine. In the western hemisphere, Savino and Rennella[4,5] in Argentina and Guida[6] in Brazil have reported porcine infection with *L. pomona*. Strains isolated by Baker and Little[7] and Reinhard, Tierney, and Roberts[8] from clinical cases of bovine leptospirosis occuring in the United States were identified as *L. pomona* at the Veterinary Division, Army Medical Service Graduate School.[9] The present paper reports what is believed to be the first bacteriologically proved leptospirosis of swine in the United States. These studies were conducted during the investigation of an explosive outbreak of disease in young shoats, characterized by marked involvement of the central nervous system.

MATERIALS AND METHODS

Herd History.—The disease outbreak studied occurred on an Ohio stock farm. The animal population consisted of 2 horses, 18 beef cattle, 22 breeding sows, and approximately 200 shoats. No dairy cattle had been kept on this farm for several years past. An unidentified mastitis had developed in the dairy herd and the owner had disposed of the animals. The horses and beef cattle were pastured separately from the swine on a field draining into a low wooded area. This wooded lowland was used as a farrowing lot. Brood sows were confined to this area. At weaning time, the shoats were segregated into three groups according to size and outward physical appearance. Each group was moved to a separate feedlot. These lots, though adjacent to each other, did not adjoin, and no direct contact existed between the three groups of shoats. At this time, two of the groups were vaccinated against hog cholera by use of antiserum and virus. The culls were not vaccinated because of their poor physical condition. Shortly thereafter, 10 shoats purchased at a stock sale were introduced into the group of culls. These animals were reportedly vaccinated with antiserum and virus prior to their purchase.

The disease outbreak appeared approximately six weeks after weaning and segregation of the shoats. It was confined to the nonvaccinated animals, all of which died of the disease within four weeks. Administration of hog cholera antiserum was without effect on the morbidity or the mortality of the disease.

Affected animals showed irregular temperature curves, inappetence, marked weakness, opisthotonos, circling, convulsions, and some became paralyzed.

Moribund animals were killed and autopsies conducted. Marked petechiation of the visceral organs and intestinal mucosa was observed, as well as evidence of pronounced diffuse nephritis.

Diagnostic Laboratory Studies.—Consideration of the herd history and of the symptoms and autopsy findings on affected animals suggested four possible etiological agents: an atypical hog cholera virus, an unknown neurotropic virus, an overwhelming bacterial toxemia, and leptospirosis.

Bacteriological examination of tissues from affected animals demonstrated systemic infection with *Salmonella choleraesuis* (*Bacillus suipestifer*). The organism was recovered from all organs cultured except the brain.

Virus studies were conducted with brain material found to be free of bacterial pathogens. Three shoats were inoculated subdurally with 0.15 ml. of a 10 per cent brain suspension from an affected animal killed in moribund condition. Two of the inoculated animals were hog cholera-susceptible; one was hog cholera-immune. The latter, aside from a transient fever during the first two days postinoculation, remained normal. Both hog cholera-susceptible pigs developed high fevers and became visibly sick by the fifth day. Symptoms exhibited were inappetence, impaired vision, incoördination, hyperesthesia, and progressive weakness. One pig died on the seventh day postinoculation,

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while the other was destroyed, in moribund condition, on the thirteenth day. On autopsy, enlargement of the spleen, diffuse nephritis, and marked edematous swelling of the submaxillary lymph glands were observed. Microscopic and cultural examination for bacterial pathogens was negative.

and the porcine strain isolated. Cross agglutination-lysis tests were conducted on these serums, using as antigens living cultures of the above-named leptospiras and, in addition, the porcine *L. pomona* strain *suis* of Savino and Rennella.‡ The results of this examination are shown in table 1.

TABLE 1—Cross Agglutination-Lysis of Porcine Leptospiras with Known Type Strains

Antigen	Antiserum against				
	Leptospira icterohaemorrhagiae	*Leptospira canicola*	*Leptospira bataviae*	*Leptospira pomona*	*Porcine Leptospira*
L. icterohaemorrhagiae	++++	+	—	+	—
L. canicola	+++	++++	—	—	—
L. bataviae	—	—	++++	—	—
L. pomona	+	—	—	++++	++++
L. pomona, (suis)	++	—	—	++++	++++
Porcine Leptospira	—	—	—	++++	++++

— = no cross reaction; + = titer less than 1/16 of the homologous strain; ++ = titer equivalent to 1/16 of the homologous strain; +++ = titer equivalent to 1/4 of the homologous strain; + + + + = titer equivalent to that of homologous strain.

Serum samples from all 4 of the affected animals submitted to the laboratory were examined for the presence of leptospiral antibodies. The microscopic agglutination-lysis test of Schuffner and Mochtar[10] was employed. All revealed antibodies against *L. pomona* in titer greater than 1 : 3,200.

Attempts to isolate leptospiras at the time that 3 of the pigs were killed were successful in but 1 case in which cultures of the urine revealed their presence. All other cultures and test animals were overwhelmed by Salmonella.

It is apparent that this strain, designated in our laboratory as S-91, is antigenically indistinguishable from either of the other strains of *L. pomona* and is completely distinct from *L. icterohaemorrhagiae*, *L. canicola*, and *L. bataviae*.

Epidemiological Survey.—Serum samples were collected from all horses, beef cattle, dogs, and breeding sows kept on this farm. In addition, ten samples were obtained from each of the nonaffected groups of shoats and from the 10 vaccinated shoats introduced into the affected group. These serums were exam-

TABLE 2—Serological Survey of Affected Farm Examination for Agglutinating-Lysing Antibody Against Leptospira Pomona (S-91)

Animals	Location	Titer		
		1 : 400 or greater	1 : 50 to 1 : 200	0
Brood sows	North pasture	13	0	8
Best shoats	West feedlot	2	0	8
Average shoats	South feedlot	8	0	2
Purchased shoats	Concrete pen*	5	2	3
Horses	East pasture	2	0	0
Beef cattle	East pasture	14	1	3
Dog	Unrestricted	0	0	1

*Site of disease outbreak.

Identification of the Leptospiral Strain.—The strain isolated was compared with strains known to exist in the United States. Antiserums were prepared in rabbits against the following leptospiras: *Leptospira icterohaemorrhagiae*, Wijnberg strain, type AB,* *Leptospira canicola*, Ruebush strain,** *L. pomona*,† *Leptospira bataviae*, Swart van Tienen strain,‡ ined for antibodies against the leptospiral strain isolated from the affected animal. The results of this examination are shown in table 2.

Serological evidence indicates extensive leptospiral infection of the domestic animals of the farm. Of significance is the absence of evidence of canine infection. Also of possible significance is the relatively low incidence of infection among the healthiest and strongest shoats (west feedlot), as compared to the high incidence in the other groups.

*Received from Prof. Dr. P. H. Van Thiel, University and Inst. of Tropical Medicine, Leiden, The Netherlands.

**Isolated at the Veterinary Division, AMSGS.

†Received from Dr. John Bozicevich, The National Institutes of Health, Bethesda, Md.

‡Received from Dr. John Bozicevich, The National Institutes of Health.

‡Received from Dr. E. Savino, Instituo Bacteriologico Malbran, Buenos Aires, Argentina.

DISCUSSION

In view of the findings reported above, it appears probable that the primary cause of disease in this herd was the virus of hog cholera, complicated by concurrent infection with *S. choleraesuis*.

Because of the presence of these two recognized pathogens, the role of leptospiras in this outbreak can not be definitely assessed. It seems reasonable, however, to postulate that leptospiral infection in the suckling pigs resulted in general unthriftiness and lowered resistance. This, coupled with exposure to hog cholera virus and *S. choleraesuis* infection, resulted in an explosive and devastating outbreak of disease. The similarity between the symptoms observed and the classical description of porcine leptospirosis by Gsell[11] is striking. However, proof of pathogenicity of this organism alone for swine must await experimental confirmation.

SUMMARY

An explosive outbreak of disease in swine is reported. In addition to the virus of hog cholera and *Salmonella choleraesuis*, *Leptospira pomona* was recovered from an affected animal.

Serological evidence indicates widespread subclinical infection among the remaining swine and other domestic animals maintained on this farm.

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Reversal of Thionine Inhibition by Various Substrates on Brucella Suis

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IN A PRELIMINARY report on the comparative effects of related chemical groups on the growth of Brucella, Cameron and Meyer[1] concluded that no consistent pattern was apparent within the groups whereby the bacteriostatic action could be predicted on the basis of chemical structure. Fischer and his associates[2] believe that the antibacterial activity of certain dyes is due to the presence of quinoid structures. They also state that no common features can be found among members of the active group. Since it has been established that enzymes are inhibited by certain dyes, it was deemed advisable to investigate this phase of the problem. Enzyme inhibition by dyes has been described by Quastel[3] and by Sevag, Gots, and Steers.[4] The work herein reported was also suggested by the findings of Lichstein and Cohen[5] who studied the transaminase reaction in several bacterial species, and Gale's[6] discussion of the intercellular utilization of glutamic acid and its inhibition by certain antibacterial agents.

In view of the preliminary findings demonstrating the variation between the species, it was deemed advisable to limit the investigations here reported to a single species, followed by comparative tests on the other two. Fromageot[7] stated that all organisms producing H_2S did so because of the presence of a desulfhydrase enzyme; *Brucella suis* is active in producing H_2S and therefore was selected as the test organism. Since one of these enzymes is specific for cysteine, and since it was necessary to establish the presence of the enzyme in the organism, the substrate was added in varying amounts to a washed suspension of *Br. suis* cells. Concurrently, the following additional substrates were also employed; glutamic acid, glutathione, and all possible combinations of the component amino acids of glutathione (cysteine, glutamic acid, and glycine). Because of its use in differentiating the species, and the fact that it possesses the quinoid structure, the effect of thionine in the presence of these substrates was also studied. In addition to the work with resting cells, the action of thionine on the growth of the organism on tryptose agar in the presence of free substrates was also studied. Cysteine could not be used in the latter study because of its incompatibility with thionine; the dye was decolorized and a precipitate formed. This report, therefore, deals with the modification of the effect of thionine on growth of *Br. suis* by the addition of various substrates, and evidence of the presence of a desulfhydrase enzyme in that species.

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METHODS

Resting Cell Investigations.—Brucella suis was grown on tryptose agar for forty-eight hours and the growth washed off with a fifteenth-molar phosphate buffer pH 6.8 to 7.0. The bacterial suspension was then washed twice and resuspended in the buffer to a concentration of 1 cc. of packed cells to 10 cc. buffer. Four cubic centimeters of the suspension were placed in 25-cc. Erlenmeyer flasks, and the desired substrate or quantity of thionine added. The quantity of H_2S produced was measured on lead acetate paper in comparing it to a standard prepared by evolving H_2S from known concentrations of Na_2S in distilled water. The method was patterned after the Gutzeit[8] technique for the quantitative determination of arsenic. The flasks were incubated in a water bath at 37.5 C. for twenty-four hours.

Growth on Nutrient Mediums.—The methods were identical to those used in the original investigations of Cameron and Meyer.[1] The various substrates were added to the mediums so that the final concentration was measured in micrograms per cubic centimeter.

RESULTS

The Effect of Glutamic Acid and Compounds Containing Glutamic Acid on the Tolerance of Brucella Suis Toward Thionine.—On tryptose agar containing 200 μg. per cubic centimeter of thionine, growth was inhibited. On the addition of glutamic

acid to the mediums containing thionine, the amount of the dye that would permit growth was markedly increased; the more glutamic acid added, the greater the quantity of thionine that could be tolerated by the organism. Three milligrams of glutamic acid per cubic centimeter of mediums would permit growth of *Br. suis* in the presence of a concentration of thionine at 600 μg. per cubic centimeter.

With glutathione, the results were similar to those obtained with glutamic acid, with the exception that a lower concentration of glutathione was necessary to counteract the effect of thionine. The results were identical when the component amino acids of glutathione were used simultaneously. Cysteine could not be used alone; glycine was inactive in counteracting the effect of thionine. In order to determine if the action of glutamic acid was specific toward *Br. suis*, the work was repeated with *Brucella melitensis* and *Brucella abortus*. In the latter two species, the effect of thionine was not counteracted by glutamic acid.

Resting Cell Investigations.—The results are summarized in table 1. The combination of resting cells and 10 mg. of cysteine produced 1.4 cc. of H_2S, a quantity much greater than with any other substrate, including glutathione. The addition of 10 mg. of thionine to the above suspension completely inhibited H_2S production. When the component amino acids of glutathione were added to the cells in amounts equivalent to these present in glutathione, the H_2S production was greater than in the combined form. When glutamic acid or glutathione was added to the combination of cysteine and thionine, a small quantity of H_2S was produced. As in the investigation with the reproducing cell, glycine was inactive under these conditions.

DISCUSSION

It should be emphasized that the method of measuring the amount of H_2S produced was semiquantitative and valuable for comparative purposes only. A variation in enzyme activity may occur between batches of organisms, and it is necessary to establish the inhibiting level of thionine for each. In the particular batch from which the results were reported, 10 mg. of thionine completely inhibited enzyme activity.

From the results with the resting cells, it is apparent that *Br. suis* contains a desulfhydrase enzyme active on substrates containing cysteine, and that the enzyme is inactivated by thionine. The question of auto-oxidation of cysteine as discussed by Hoyer[9] was considered following the investigations on *Br. suis*. In comparing the results with those obtained from *Br. melitensis*, it was apparent that the amount of enzyme varied between the species. Had auto-oxidation alone been involved, the results should have been identical in all species.

Glutamic acid undoubtedly plays an important role in the metabolism of *Br. suis*, as demonstrated by the fact that it counteracted the inhibitory action of a concentration of thionine which would not permit

TABLE 1—The Inhibition by Thionine of Hydrogen Sulfide Production from Cysteine by Resting Brucella Suis

Cells	Substrate	Inhibitor	H_2S produced
Brucella suis	.....	.....	0.035 cc.
.....	10 mg. cysteine in buffer	.....	0.050 cc.
.....	20 mg. cysteine in buffer	.....	0.070 cc.
Br. suis	10 mg. cysteine	.....	0.770 cc.
Br. suis	20 mg. cysteine	.....	1.120 cc.
.....	20 mg. glutathione	.....	0.020 cc.
Br. suis	10 mg. glutathione	.....	0.210 cc.
Br. suis	20 mg. glutathione	.....	0.290 cc.
Br. suis	10 mg. uncombined glutathione	.....	0.490 cc.
.....	20 mg. taurine in buffer	.....	.....
Br. suis	20 mg. taurine	.....	.....
.....	20 mg. dl-methionine in buffer	.....	.....
Br. suis	20 mg. dl-methionine	.....	.....
Br. suis	10 mg. cysteine	5 mg. thionine	0.250 cc.
Br. suis	10 mg. cysteine	10 mg. thionine	.....
Br. suis	{10 mg. cysteine {10 mg. glutamic acid	10 mg. thionine	0.230 cc.
Br. suis	10 mg. cysteine 20 mg. glutamic acid	10 mg. thionine	0.200 cc. .....
Br. suis	{10 mg. cysteine {20 mg. glutathione	10 mg. thionine	0.040 cc.
.....	20 mg. cysteine	.....	0.070 cc.
Brucella abortus	20 mg. cysteine	.....	0.910 cc.
Brucella melitensis	20 mg. cysteine	.....	0.480 cc.

growth on tryptose agar. In the other two species, glutamic acid failed to increase their tolerance to thionine. Apparently, glutamic acid does not counteract the inhibitory action of thionine on desulfhydrase activity in *Br. suis*, as evidenced by the investigations with resting cells in the presence of thionine and glutamic acid wherein no H_2S was produced. Yet, glutamic acid stimulated growth when added to mediums containing a concentration of thionine that would not permit growth in the absence of glutamic acid. Apparently, *Br. suis* growth will occur independent of H_2S production, an adaptive feature already described by Dubos.[10] It is unfortunate that, because of an incompatibility, we could not duplicate with cysteine the action of glutamic acid in counteracting thionine. Although it could not be measured, it was apparent that glutathione, a compound containing both glutamic acid and cysteine, produced significantly greater growth in the presence of thionine than did glutamic acid alone. No attempt is made at this time to explain the selective action of glutamic acid towards *Br. suis*, an action not apparent towards the other species.

SUMMARY

The inhibition of a desulfhydrase enzyme, apparently a component of Brucella, by thionine is described. Glutamic acid did not counteract this inhibition either with the resting, or reproducing, cell. The amino acid, on the other hand, counteracted the inhibitory effect of thionine of the growth of *Brucella suis*.

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Studies on Baby Pig Mortality
VIII. Chemical Observations on the Newborn Pig, with Special Reference to Hypoglycemia

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In 1941, Graham, Sampson, and Hester[12] described a fatal condition in newborn pigs in Illinois characterized by acute hypoglycemia. In 1942, Sampson, Hester, and Graham,[21] after further study of the syndrome, demonstrated that a fatal hypoglycemia could readily be produced in newborn pigs by fasting. The results of additional study of the relation of age to fasting hypoglycemia in newborn pigs were reported by Hanawalt and Sampson[16] in 1947. These studies showed that pigs subjected to fasting twelve to twenty-four hours after farrowing are highly susceptible to the development of intense hypoglycemia, while those which are properly nourished for five to six days are less susceptible but not entirely refractory to fasting hypoglycemia thereafter. The symptoms accompanying the experimentally produced fasting hypoglycemia were indistinguishable from those seen in naturally affected pigs. They included dullness, shivering, inappetence, weakness, rough hair coat, somnolence, hypothermia, and, ultimately, coma. Gross pathological lesions were absent except for the lack of evidence of ingesta in the gastrointestinal tracts of many of the affected pigs. The primary significance of the hypoglycemia was further suggested when it was demonstrated that some of the naturally affected pigs in the early stages of the malady, as well as the hypoglycemic-fasted pigs, responded favorably to repeated injections of glucose.

In 1944, Madsen, Earle, Heemstra, and Miller[21] reported rather heavy losses among baby pigs farrowed at the Beltsville Research Center, Beltsville, Md., and surrounding area. Efforts to demonstrate the presence of an infectious disease were unsuccessful, and spectrophotometric analyses of feed and water given to the sows and of bones, liver, and urine of affected pigs failed to demonstrate the presence of toxic quantities of chemical poisons such as lead, arsenic, mercury, or fluorine. Several symptoms in affected animals apparently resembled those described by the Illinois workers; however, blood sugar levels even in very sick pigs were reported as often within the normal range. Furthermore, appreciable clinical response did not follow intraperitoneal injection of calcium gluconate, dextrose, or thiamine, or hand-feeding with cows' milk containing 10 per cent dextrose. Autopsy findings were described as usually variable or negative, except for the rather constant presence of an orange to white precipitate in the renal papillary ducts, renal pelves, ureters, or urinary bladder. The increase in nonprotein nitrogen values found in the blood of affected pigs, particularly of the urea and uric acid fractions, and the determination that the precipitate found in the urinary tract consisted largely of uric acid or urates led the authors to believe that "—the cause of death in these baby pigs was associated with acute uremia."

In 1946, Doyle and Hutchings[9] described a transmissible gastroenteritis in pigs characterized by diarrhea, vomiting in some cases, rapid loss of weight, and a high death rate among the very young pigs. The most evident gross lesions, when present, were gastritis, enteritis, engorgement of mesenteric vessels, the presence of degenerative changes and urates in the kidneys, and a large amount of highly fluid intestinal content having a whitish, yellowish, or greenish color.

In 1949, Green, Kernkamp, Roepke, and Winters[13] reported a "severe and often fatal metabolic disturbance" in baby pigs fed chiefly on fat-free milk solids reconstituted with water. The disturbance was described as displaying "the characteristics of a toxemia that was manifested by nausea, vomiting, diarrhea, dehydration, itching, muscular jerks, stupor, and coma." Associated with the syndrome was a high degree of nitrogen retention in the blood, which the authors designated a uremia.

In 1951, Grace, Morrill, Butzow, Hendren, and Sampson[11] described blood and tissue changes in a true uremia experimentally produced in pigs by obstructing urine flow through ligation of the ureters. They suggested the advisability of clarification of the concept of what constitutes a true uremia and of dif-

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TABLE I—Results of Body Fluid and Tissue Analyses on Newborn, Suckled, and Fasted Pigs and Clinical Cases

		Group 1			Group 2			Group 3			Group 4		
		Newborn Pigs			Suckled pigs			Fasted pigs			Clinical cases		
		Mean	Range	No.*	Mean	Range	No.*	Mean	Range	No.*	Mean	Range	No.*
Analyses on blood													
Hemoglobin	Gm. %	10.0	5.6-13.5	26	8.2	4.9-11.8	33	11.5	6.6-17.5	44	9.1	7.5-13.5	22
Sugar (glucose)	mg. %	102.6	49.72-158.2	26	114.2	74.58-144.64	33	{ 15.6 { tr.**	2.26-47.46	{ 18 { 27	28.3	tr.-101.7	22
Nonprotein nitrogen	mg. %	44.6	29.74-73.71	26	66.4	45.20-123.19	33	81.4	42.32-155.34	44	109.9	48.0-277.3	22
Urea nitrogen	mg. %	22.3	10.4-49.0	26	30.8	10.1-69.0	33	48.4	10.1-138.5	43	65.8	16.3-262.1	20
Creatinine	mg. %	2.3	1.36-4.31	25	1.5	0.96-2.15	33	1.7	0.36-2.85	43	2.0	0.97-5.10	21
Uric acid	mg. %	1.4	0.27-3.60	26	0.8	0.0 (tr.)-2.15	33	1.4	0.0-3.60	44	2.5	0.41-10.48	21
Chlorides	mg. %	476.2	425.0-492.0	13	485.1	470.0-490.0	23	482.0	455.0-496.0	29	483.6	475.0-490.0	11
Analyses on serum													
Calcium	mg. %	10.6	8.0-12.5	25	12.0	7.5-14.5	33	10.3	7.5-14.5	39	11.1	8.0-14.5	19
Phosphorus	mg. %	7.4	5.23-9.50	19	7.4	5.88-8.80	27	9.0	5.9-11.5	18	6.6	2.94-11.5	15
Magnesium	mg. %	2.2	1.38-3.00	15	2.5	1.69-3.20	13	3.1	1.88-4.57	15	2.5	2.01-3.00	3
Analyses on urine***													
Total nitrogen	mg. %	494.0	132.0-925.0	8	1039.0	260.0-2212.0	22	1017.0	328.0-2128.0	23	1156.0	322.0-1860.0	15
Ammonia nitrogen	mg. %	54.8	31.7-78.4	4	50.8	9.4-116.6	14	97.4	25.3-397.0	12	53.8	9.7-127.6	11
Uric acid	mg. %	29.4	3.2-59.2	7	21.0	6.1-55.8	22	65.0	24.5-126.1	23	191.5	27.8-655.2	15
Analyses on tissues													
Liver glycogen	%	5.2	1.85-9.00	14	2.7	tr.-6.78	26	tr.	tr.	25	tr.	tr.-0.16	18
Kidney uric acid	mg./100 Gm.	5.2	0.0-14.80	9	4.0	0.0-20.79	28	11.8	0.0-78.06	31	36.7	0.0-189.93	20

*Number of pigs on which data are included. **tr. = trace. ***These data are based on random samples obtained at autopsy and hence can not be considered strictly quantitative. % = per 100 ml.

ferentiating between true uremia and azotemia of prerenal or extrarenal origin.

PURPOSE OF THIS INVESTIGATION

This experiment was designed with a twofold purpose: *viz.*, to study (1) the chemical alterations in some of the body tissues and fluids in newborn pigs under conditions of fasting and in certain naturally occurring conditions resulting in death, and (2) the histopathological alterations, if any, in the same or similarly affected pigs. Results of the chemical studies are reported herein; the morphological observations form the basis of a separate report.

EXPERIMENTAL PROCEDURES

Experimental Animals and Their Management.—Except in cases with naturally acquired or spontaneous hypoglycemia (hereafter referred to as clinical cases), all pigs used in this study were from gilts and sows of predominantly Hampshire breeding. Breeding animals were kept under drylot conditions and fed a basal ration consisting of 60 per cent ground yellow corn, 25 per cent ground oats, 5 per cent tankage, 5 per cent soybean oil meal, 4 per cent alfalfa meal, and 1 per cent mineral. The mineral was a mixture containing two parts steamed bone meal, two parts ground limestone, and one part iodized salt. In addition, alfalfa hay was available in racks most of the time. In some cases, additional corn and soybean oil meal were supplied, but always in small amounts and in such proportion as would at least maintain the protein content of the ration. The animals were hand-fed at a level of approximately 2 lb. of feed per 100 lb. of body weight per day.

A few days prior to farrowing, each sow was isolated in a pen bedded with wood shavings (fig.1). Except in warm weather, farrowing occurred in a room steam-heated to approximately 70-75 F. to prevent chilling of the pigs. Pigs to be fasted were taken from the sow as soon as possible after farrowing, usually before they had suckled. For the chemical studies, litters were divided as evenly as possible on the basis of sex and body weight into three groups of 26, 33, and 46 pigs, respectively. Group 1 (26 animals) was sacrificed as normal newborn pigs one to fifteen hours after farrowing. Group 2 (33 animals) was suckled by the dams for fifteen to one hundred and fifty hours, to constitute additional normal control pigs. Groups 3 (46 animals) was taken from the dams soon after farrowing, fasted completely, and sacrificed when moribund. Group 4 was made up of 23 cases of naturally occurring so-called "baby pig disease," in most of which hypoglycemia was a prominent feature.

Experimental Procedures with Animals.—Pigs which were fasted (group 3) and those with naturally acquired disease (group 4) were allowed to reach an extremely weakened or moribund condition. All were killed by exsanguination. Blood samples were collected over potassium oxalate for determinations to be made on whole blood or blood filtrate, and without an anticoagulant for determinations to be made on serum. Autopsy was performed immediately, and approximately 2-Gm. samples of liver were placed in 30 per cent potassium hydroxide within about two minutes from the time the animal was exsanguinated. Urine, if present in significant amount, was withdrawn from the bladder by means of clean glass syringe and needle.

Chemical Methods.—Chemical Determinations on Blood.—Hemoglobin determinations were made by the acid-hematin method and measured with a Fisher electrohemometer. Unusually high or low values were checked by the method of Newcomer.[23] Blood sugar analyses were made by the Shaffer-Hartman-Somogyi method according to the technique of Koch.[13] Nonprotein nitrogen determinations were made by the Koch-McMeekin micro-Kjeldahl method as applied to estimation of nonprotein nitrogen in blood filtrates (direct nesslerization).[16] Urea nitrogen estimations were made by Karr's direct nesslerization method for urea.[15] The Folin method for estimation of creatinine in blood according to Koch[10] was employed. The readings were made on a Klett-Summerson photoelectric colorimeter. Blood-uric acid content was determined by the method of Folin,[5, 9] and these readings also made with a Klett-Summerson photoelectric colorimeter. Blood chlorides were determined by the method of Whitehorn.[26]

Chemical Determinations on Blood Serum.—Serum calcium was measured by the Clark and Collip modification of the Kramer-Tisdall method.[3] Serum inorganic phosphorus determinations were made by Youngburg's system for partition of blood phosphorus.[27] Serum magnesium was measured by the method of Denis[5] modified according to Hawk and Bergeim.[17]

Chemical Determinations on Urine.—Total nitrogen in the urine was determined by the Koch-McMeekin micro-Kjeldahl method as applied to urine (direct nesslerization).[20] The ammonia-nitrogen determinations were made by the permutit or scolite method for ammonia in urine as described by Folin and Bell.[10] Urinary uric acid was measured by the Folin direct colorimetric method as set forth by Simmons and Gentskow.[25] Equipment which would have made possible the collection of 24-hour specimens of urine arrived too late for the experiment; therefore the data obtained can not be considered strictly quantitative in character.

Chemical Determinations on Tissues.—Liver glycogen was determined by the technic of Blatherwick, Bradshaw, Ewing, Larson, and Sawyer.[2] For estimation of uric acid in kidney tissue, a kidney extract was made according to the technique described by Madsen, Earle, Heemstra, and Miller,[21] and the determination made by the Folin direct colorimetric method.[5, 9]

RESULTS

The results of biochemical determinations on the four groups are summarized in table 1. The results of determinations on newborn and suckled pigs (groups 1 and 2) are considered as indicating the normal status or trend of the various body fluid and tissue components studied, during the first few days of life.

Fasted pigs and clinical cases presented rather strikingly similar pictures, differing from normal control pigs in the same respects and often in similar degree. In both the fasted pigs and clinical cases, symptoms included dullness, shivering, weakness, rough hair coat, somnolence, hypothermia, and, ultimately, coma (fig. 2). Among the body fluid and tissue constituents studied, the greatest differences between normal and affected pigs appeared in concentrations of glucose and nonprotein nitrogenous compounds of blood, glycogen content of liver, and uric acid content of kidneys.

Blood Sugar.—In 26 newborn pigs, blood sugar concentrations ranged from 49.72 to 158.20 with an average of 102.6 mg. per 100 ml. of blood. Among 33 suckled pigs left with the sows, the concentrations ranged from 74.58 to 144.64 and averaged 114.2 mg. per 100 ml. of blood. Values for 27 of the fasted pigs were so low as to read approximately zero and were recorded as a trace only; in 18 cases for which numerical values were recorded, the range was from 2.26 to 47.46 and the average, 15.6 mg. per 100 ml. of blood. Among 22 clinical cases, the range was from a trace only to 101.7 mg. per 100 ml. of blood; however, excluding the two highest readings, the next highest reading was 33.90 mg. per 100 ml. of blood.

Nonprotein Nitrogen.—In 26 newborn pigs, nonprotein nitrogen concentrations ranged from 29.74 to 73.71 and averaged 44.6 mg. per 100 ml. of blood. The 33 suckled pigs ranged from 45.20 to 123.19 and averaged 66.4 mg. per 100 ml. of blood. The range in 44 fasted pigs was 42.32 to 155.84 and averaged 81.4 mg. per 100 ml. of blood. Twenty-two clinical cases showed a range of

48.0 to 277.3 and an average of 109.9 mg. per 100 ml. of blood.

Urea Nitrogen.—That a considerable proportion of the increase in nonprotein nitrogen in fasted pigs and clinical cases may have been due to an increase in the urea nitrogen fraction was suggested by the results of these determinations. The 26 newborn pigs showed a urea nitrogen range of 10.40 to 49.0 and an average of 22.3 mg. per 100 ml. of blood. The 33 suckled pigs ranged from 10.1 to 69.0 and averaged 30.8 mg. per 100 ml. of blood. Forty-three fasted pigs ranged from 10.1 to 138.5 and averaged 48.4 mg. per 100 ml. of blood. Twenty clinical cases revealed a range of 16.3 to 263.1 and an average of 65.8 mg. per 100 ml. of blood.

Liver Glycogen.—Fourteen newborn pigs showed liver glycogen concentrations of 1.85 to 9.00 per cent, averaging 5.2 per cent on a fresh basis. Among 26 suckled pigs, values ranged from a trace (in 4 cases) to 6.78 per cent and averaged 2.7 per cent. No fasted pig and only 1 clinical case showed more than a trace of hepatic glycogen. For the latter pig, a value of 0.16 per cent was recorded.

Kidney Uric Acid.—Some pigs in each of the four groups showed as little as a trace only of uric acid. The highest values obtained among 9 newborn, 28 suckled, and 31 fasted pigs, and 20 clinical cases were 14.8, 20.79, 78.06, and 189.93 mg. per 100 Gm. of tissue, respectively, on a fresh basis. The averages for the same groups were 5.2, 4.0, 11.8, and 36.7 mg. per 100 Gm. of tissue, respectively.

Other minor differences in the body fluid and tissue components were observed which may conceivably be explained on the basis of complicating factors such as dehydration (*see* Discussion).

DISCUSSION

In accord with the findings of Hamilton *et al.*[14, 15] and Craft and Moe,[4] the results of this study indicate that hemoglobin concentration normally declines somewhat during the first three or four days of life. In the fasted pigs, on the other hand, hemoglobin concentrations were generally higher than in the strictly newborn animals. The explanation may lie in the degree of dehydration and resulting hemoconcentration existing in the fasted pigs.

Results of work reported by Sampson, Hester, and Graham[24] and Hanawalt and Sampson[16] on the susceptibility of newborn pigs to hypoglycemia from fasting were confirmed. It is evident that pigs are usually born with an appreciable supply of reserve carbohydrate, the liver glycogen concentration apparently being nearly twice as high at birth as it is three to five days later. It appears, however, that this store is rapidly depleted unless a means of replenishment is readily available. When it is

Fig. 1—The pigs were predominantly of the Hampshire breed and were farrowed in pens bedded with wood shavings. Except in warm weather, it was arranged for farrowing to occur in a steam-heated room to avoid possible chilling of pigs.

realized that baby pigs may double their birth weight in one week, the reason for their prodigious appetites and the frequent urge to satisfy them is evident.

Concentrations of nonprotein nitrogen and urea nitrogen in the blood were significantly increased in fasted pigs and clinical cases (groups 3 and 4) as contrasted with values for suckled pigs. For several reasons, however, it is believed that hypoglycemia rather than azotemia was responsible for the syndrome observed. (1) Intense hypoglycemia was present, and it is known to be capable of causing most if not all of the symptoms observed. Furthermore, hypoglycemic, but not uremic, coma can often be alleviated by injecting glucose. (2) Concentrations of various nonprotein nitrogenous components of the blood for which determinations were made were still far below levels considered necessary for toxic effects in other species.[1] (3) Clinical evidence of anuria was not observed. (4) Blood chloride and serum calcium levels did not appear to be significantly lowered, as is described in true uremia of man. However, fasted pigs (group 3) did show an unexplained increase in serum inorganic phosphorus values, which has been found in uremia and is believed to be responsible for symptoms of irritability seen in that syndrome. The mechanism involved is thought to be through lowering of the calcium level. In this experiment, symptoms of irritability did not present themselves nor were serum calcium levels lowered. (5) Blood creatinine levels, as a measure of kidney function, were significantly above 3 mg. per 100 ml. of blood in only 3 instances; one of the clinical cases (group 4) showed a level of 5.1 and 2 of the supposedly normal newborn pigs (group 1) had levels of 3.21 and 4.31 mg. per 100 ml. of blood, respectively.

In view of these observations, it is believed that the syndrome developing as a result of fasting can be best explained on the basis of the hypoglycemia and that the rather mild increase in nonprotein nitrogenous components of the blood represents an extrarenal azotemia associated with increased protein catabolism or dehydration, or both, rather than true uremia.

Data on urinary nitrogen, while suggesting strongly an increase in ammonia-nitrogen elimination in fasted pigs, can not be considered quantitative in character since 24-hour samples were not obtained. Since the chief source of uric acid is the purine bases which result from hydrolysis of the nucleic acids,[23] the increase in uric acid of the blood, urine, and kidney in clinical cases suggests that tissue breakdown may have been a little greater in those cases than in experimentally fasted pigs. Sampson, Hester, and Graham[24] and Hanawalt and Sampson[16] observed that the pig at 5 to 6 days of age is more refractory to fasting hypoglycemia than the newborn pig, suggesting that the older pig is better able to derive energy from the breakdown of his own

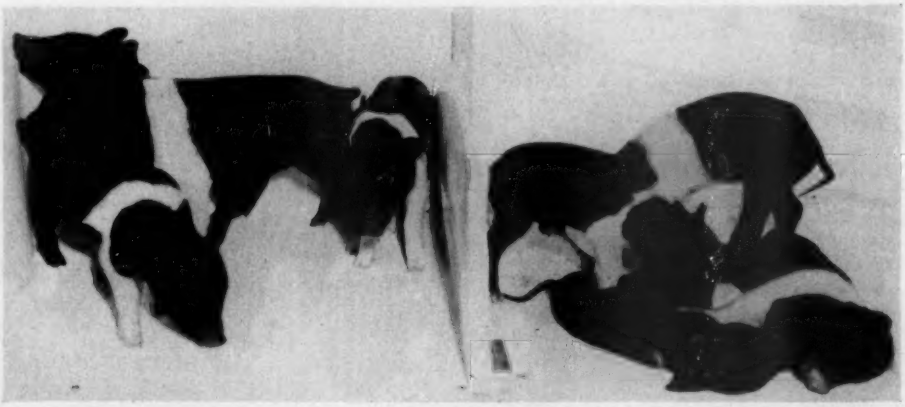

Fig. 2—Of these 8 litter mates, the 4 on the left were left with the sow while those on the right were fasted. Two of the fasted pigs are comatose and the other 2 are weak and somnolent after seventy hours at room temperature. The pigs allowed to nurse the sow remained apparently healthy and gained weight rapidly.

tissues. Since the average age of clinical cases was approximately 86.5 hours as compared to 42.7 hours for experimentally fasted pigs, life of the former probably having been prolonged by obtaining small amounts of milk, it is possible that the higher uric acid values were due to a slightly increased efficiency in breaking down body tissue for energy purposes on the part of the older pigs.

Further investigation of some aspects of spontaneous hypoglycemia in pigs seems desirable. For example, additional data are needed on effects of dehydration and data on concentrations of free phenols, guanidines, or other possible toxic substances in the blood of comatose pigs. From a practical standpoint, it would seem desirable to determine if possible that degree of hypoglycemia at which irreparable damage to the central nervous system is likely to occur and whether hypothermia or other readily measurable effects can be used as a guide in treatment and prognosis. Studies on basal metabolic rates might well be included. Also, since gluconeogenesis is stimulated by certain adrenocortical substances,[7] it would be of interest to determine the effect, if any, of adrenal cortical hormones upon the carbohydrate metabolism of fasting newborn pigs.

SUMMARY AND CONCLUSIONS

Chemical observations on 128 baby pigs are presented. Included are data obtained on 26 apparently normal newborn pigs destroyed for study between one and fifteen hours after birth, 33 apparently normal pigs allowed to suckle their dams for fifteen to 150 hours, 46 pigs fasted until moribund, and 23 pigs affected with a naturally occurring syndrome which clinically resembled that produced by fasting.

Chemical determinations included those for hemoglobin, glucose, nonprotein nitrogen, urea nitrogen, creatinine, uric acid, and chlorides in whole blood; calcium, inorganic phosphorus, and magnesium in blood serum; total nitrogen, ammonia nitrogen, and uric acid in the urine (only random or final samples); glycogen content of liver; and uric acid content of kidney.

The results appear to justify the following conclusions:

1) The normal newborn pig has an appreciable store of carbohydrate as indicated by the results of chemical determination of blood sugar and hepatic glycogen concentrations.

2) Under conditions of fasting during the first few days of life and in certain naturally occurring syndromes, the hepatic glycogen is rapidly depleted and severe and fatal hypoglycemia readily develops.

3) Carbohydrate depletion is accompanied by significant increases in blood nonprotein nitrogen and urea nitrogen, and sometimes by moderate increase in uric acid content of the kidney. However, within the limits of this experiment, no evidence was obtained which would indicate that the observed azotemia resulted from disturbed kidney function.

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Studies on Baby Pig Mortality
IX. Some Morphological Observations on the Newborn Pig, with Special Reference to Hypoglycemia

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THE PURPOSE OF this paper is to report certain morphological observations made in connection with an experiment which also included chemical studies on normal and hypoglycemic newborn pigs. The outline of the main experiment and results of certain chemical studies have already been reported.[11] In view of the absence of consistent or characteristic pathological changes in both experimental and naturally occurring hypoglycemia observed by Graham *et al.*[6] and Sampson *et al.*,[14] the histopathological study was approached with the hope that it might reveal changes invisible on gross examination which would contribute to an understanding of the pathogenesis of the syndrome.

Experimental Procedure

Experimental animals and their management were the same as those on which chemical observations have been reported by the author,[11] the pigs consisting of four groups. Group 1 consisting of 29 normal newborn pigs was killed one to fifteen hours after birth. Group 2 (40 pigs) was left with the dams for fifteen to 150 hours to constitute additional normal control pigs. Group 3 (55 pigs) was taken from the dams soon after farrowing, fasted completely, and killed when moribund. Group 4 was made up of 23 pigs with naturally occurring syndromes in which hypoglycemia was a prominent feature. The fact that a larger number of pigs is included in this report than in the chemical studies[11] is because of the loss of some blood samples through clotting or death of the pigs before samples could be obtained for analysis. Procedures employed, except for histopathological methods, have already been reported.[11]

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From a portion of a thesis submitted to the Graduate College, University of Illinois, in partial fulfillment of the requirements for the degree of doctor of philosophy.

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Histopathological Methods

Tissues saved for section included liver, kidney, pancreas, adrenal, thyroid, hypophysis, cerebrum, heart, stomach (fundus), duodenum and jejuno-ileum. Liver and kidney were fixed in 10 per cent formalin, absolute alcohol, and Zenker's or Helly's fluid. Pancreas was fixed in formalin and Bouin's or Helly's fluid. The hypophysis was fixed in Bouin's or Helly's fluid; the adrenal in Bouin's, Zenker's, or Helly's fluid; and the thyroid, brain, heart, stomach, duodenum, and jejuno-ileum in Zenker's or Helly's fluid. Zenker's, Helly's, and Bouin's fluids were made up according to Mallory.[8] Tissues fixed in these fluids were washed in running water, dehydrated in alcohol, cleared in oil of cedarwood, and embedded in tissuemat.

The hematoxylin-eosin-staining procedure was applied to all tissues fixed in Zenker's, Helly's, or Bouin's fluid. After removal of tissuemat and fixative salts, such tissues were overstained in Harris' hematoxylin; differentiated in acid-alcohol; counterstained in alcoholic eosin solution; dehydrated in alcohol; cleared in xylol; and mounted in clarite. The chrome hematoxylin-phloxine technique of Gomori,[4] and Heidenhain's aniline-blue stain according to Mallory,[8] were applied to Bouin- or Helly-fixed pancreas and hypophysis and to Bouin-, Helly-, or Zenker-fixed adrenal. Alcohol-fixed liver was cleared in oil of cedarwood, embedded in tissuemat, then treated according to Best's carmine method as modified by Chipps and Duff[3] to demonstrate the presence of glycogen; similarly, alcohol-fixed kidney was subjected to the Schultz-Schmidt method as outlined by Mallory[8] to demonstrate monosodium urate and uric acid. Formalin-fixed liver and kidney from selected cases were sectioned by the frozen section method and stained with Sudan IV and Harris' hematoxylin to determine the presence of fat.[8] Sections of pancreas, hypophysis, and adrenal for the chrome hematoxylin-phloxine, hematoxylin-eosin, and aniline-blue techniques were made at a thickness of 4μ; other paraffin sections were made at 6 or 7μ.

Results

It is beyond the scope of these studies to describe completely the morphological pic-

ture of the tissues saved from all four groups of pigs. Rather, only those macroscopic and microscopic features which appeared to be of interest, or to indicate a deviation from the normal, are described or illustrated.

Heart.—No significant differences between any of the 4 groups of pigs were noted. Cross-striation of muscle fibers at these stages was barely discernible. Occasional mitotic figures were observed among the muscle cell nuclei.

Thyroid Gland.—Morphological variations in the thyroid gland concerned the relative amount of colloid present and the height of alveolar epithelium. Newborn and suckled pigs showed a moderate amount of colloid, and the epithelium might be described as of low, simple, columnar type or slightly higher than a strictly cuboidal type. More plentiful colloid of a more deeply staining character was present in most of the sections from the fasted pigs and clinical cases. In these cases, the epithelium was slightly lower or of a more distinctly cuboidal character, some even being of a low cuboidal type.

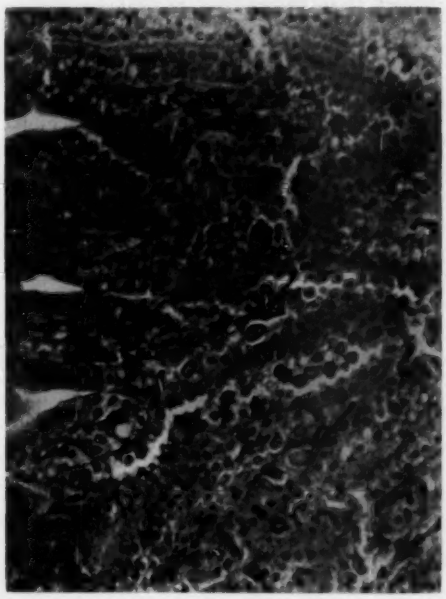

Fig. 1—Cross section of jejunum from a newborn pig (H35367) which had suckled, showing the eosinophilic inclusions in the epithelial cells of the mucosa. Hematoxylin-eosin. x 225.

Stomach.—No significant variations were noted in the stomachs of pigs of the various groups.

Small Intestine.—No significant differences were noted in the duodenum; however, in the majority of suckled pigs, and in 4 of the newborn pigs, numerous strongly eosinophilic inclusions of variable size were noted in the epithelial cells of the jejunoileum, situated generally between the nucleus and the base of the cell (fig. 1). These inclusions were especially large and prominent in the case of the 4 newborn pigs. Examination of the clinical and pathological data revealed that these 4 pigs had had ample opportunity to suckle, and that their digestive tracts contained milk at the time of autopsy. This type of inclusion was not observed in the fasted pigs or clinical cases (*see* Discussion).

Cerebrum. — No significant variations were noted.

Liver.—In the newborn and suckled pigs, the hepatic cells were distended to the extent that the sinusoids of the liver were practically obliterated. In sections stained with hematoxylin and eosin, the cytoplasm presented a foamy-to-flocculent appearance with large clear areas in evidence (fig. 2). Sections stained with Best's carmine revealed the presence of glycogen (fig. 4 and 5); however, many such sections failed to show concentrations which correlated well with results of chemical analysis. In addition to the difficulties involved in retaining glycogen in tissues during histological procedures, the sample examined is much smaller than is used in chemical analysis. These features, together with the fact that the human eye can not compare with the analytical balance as a measuring device, readily explain the limitations of the histological procedure.

In sections prepared from the livers of fasted pigs and clinical cases, and stained with hematoxylin and eosin, the hepatic cells were not thus distended, the cytoplasm being much more dense and the sinusoids clearly in evidence (fig. 3). Sections from these pigs, stained by Best's method, failed to reveal evidence of the presence of glycogen (fig. 6).

Sections prepared by the frozen section method and stained with Sudan IV and hematoxylin revealed rather numerous fine globules of lipoidal material in the livers of newborn and suckled pigs, particularly near

Fig. 2—Section from liver of newborn pig showing "foamy" character of cytoplasm in well-distended hepatic epithelial cells. Arrows indicate foci of nucleated cells which represent remnants of the earlier hematopoietic function of the liver. Hematoxylin-eosin. x 225.

the cell margins bordering on the sinusoids. Similar preparations from fasted pigs showed little evidence of such material, most of them being essentially devoid of it. There was no clear evidence of the presence of fatty metamorphosis in the livers of completely fasted pigs. However, among 19 clinical cases thus examined, 15 showed a slight to moderate degree of fatty infiltration or degeneration. Of the 15 pigs, the change was more prominent in the peripheral parts of the liver lobules in 9; it was diffuse in 4, and more prominent in the central parts of the lobules in 2 cases.

It may be of interest that the rather numerous small foci of cells and the megakaryocyte-like cells, which are present in the liver during fetal life, decreased rapidly during the first seventy-two to ninety-six hours after birth.

Kidney.—One macroscopic finding of interest was the constant presence of multiple, fine petechial hemorrhages on the surfaces of the kidneys in the pigs of all groups (*see* Discussion).

Results of examination of the kidneys for crystals of urates are shown in table 1. The cut surface of the normal kidney is seen in fig. 7, while that of one containing a concentration of crystals designated as + + + is shown in fig. 8.

On histopathological examination, the cytoplasm of epithelial cells of the convoluted tubules of kidneys from many newborn pigs and clinical cases appeared pale to almost vacuolate, not unlike the hepatic epithelium. Sudan IV preparations indicated that some lipoidal material was present. Similar preparations made from fasted pigs were negative for lipoidal material; however, of 19 clinical cases examined, only 3 were essentially devoid of fat or fatlike substances, the remainder showing from slight to marked lipoidal content in the epithelial cells of convoluted tubules.

Among fasted pigs and clinical cases, some of the larger collecting tubules appeared slightly dilated, possibly as a result of the presence of uric acid or urate crystals. However, there was no evidence of obstruction as judged by lack of dilatation of

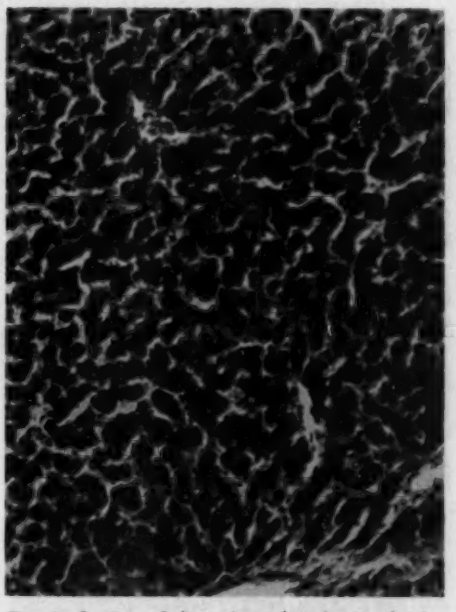

Fig. 3—Section of liver from fasted pig showing smaller hepatic epithelial cells with more densely stained cytoplasm, indicating loss of glycogen. Hematoxylin-eosin. x 225.

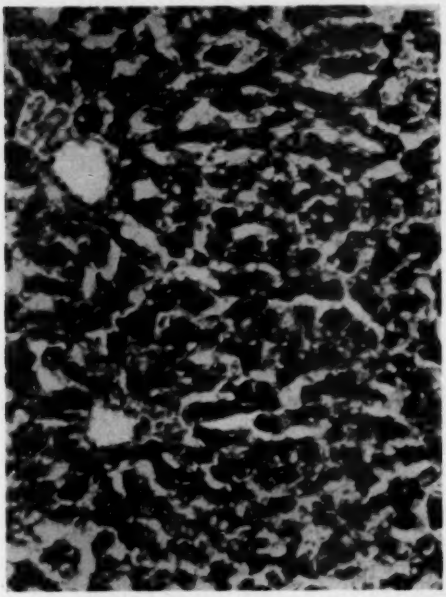

Fig. 4—Section of liver from newborn pig (H29066). Note glycogen content as contrasted with fig. 6. Best's carmine. x 225.

Fig. 5—Section of liver from suckling control pig (H29070). Note glycogen content as contrasted with fig. 6. Best's carmine. x 225.

convoluted tubules proximal to these. The Schultz-Schmidt preparations revealed the presence of occasional urate casts (fig. 9), but only in certain ones of the cases designated as + + to + + + + on macroscopic examination.

Adrenal Glands.—No variations of significance were noted.

Hypophysis.—The only variations of significance observed in the hypophyses were related to the ratio of various cell types. For a most accurate determination of cell ratios, serial sections throughout the gland, from which representative cell counts could be made, would appear to be necessary. Such

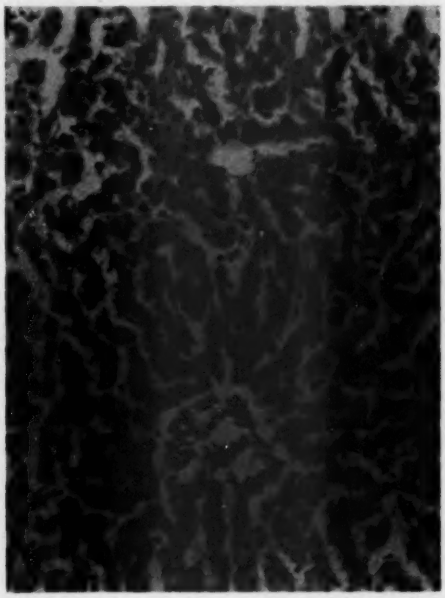

Fig. 6—Section of liver from fasted pig (H35370). Note lack of glycogen as contrasted with fig. 4 and 5. Best's carmine. x 225.

a procedure was considered beyond the scope of the present study; however, by sectioning the glands in approximately the same plane but at different levels, and noting the distribution of the various cells in those levels, it is believed that a fairly accurate conception of the picture was obtained. On the basis of such a procedure, it appeared that the proportion of *alpha* cells in the suckled pigs was silghtly greater than that in pigs of other groups. It also apppeared that the chromophobe cells were relatively

less numerous in the suckled pigs than in any of the others. Differences in proportions of *beta* cells in the different groups were not readily apparent. It must be admitted that, without benefit of actual cell counts, less obvious changes may have passed unnoticed and what appeared to be slight variations may have been misleading.

The Mallory-Heidenhain stain appeared to be superior to the hematoxylin-eosin or chrome hematoxylin-phloxine stains in demonstration of the various cell types of the hypophysis, although fairly good differentiation was obtained with all three.

Pancreas.—The distribution and size of islets appeared to be erratic in all groups; they were plentiful in some areas of the gland and scarce in others. One was left with the impression that the islet tissue was about as plentiful in one group as another. Differentiation of cell types within the islets was not accomplished well with the Mallory-Heidenhain technique; the chrome hematoxylin-phloxine stain provided fair differentiation. Gomori,[4] working on the human pancreas, evidently was able to obtain thinner and more uniform sections than was the case in this study, since he describes findings in serial sections of a thickness of 2 μ. In sections of that character, differentiation would undoubtedly be better than was obtained in this study. An effort was made to determine the proportion of *beta* cells, which are evidently related to insulin production and thus might have had an influence on the hypoglycemia.[2] Although counts of 1,000 islet cells were made in each group of pigs, *viz.*, the newborn, suckled, and fasted pigs, and in the clinical cases with the designation of 70, 69.6, 75.3, and 78.5 per cent, respectively, as *beta* cells, accuracy of the designation was questionable in some cases. Thus, it can only be stated that, under the conditions of this study, no variations of significance were noted between the various groups.

It was observed, also in agreement with Gomori's findings in man, that there is a

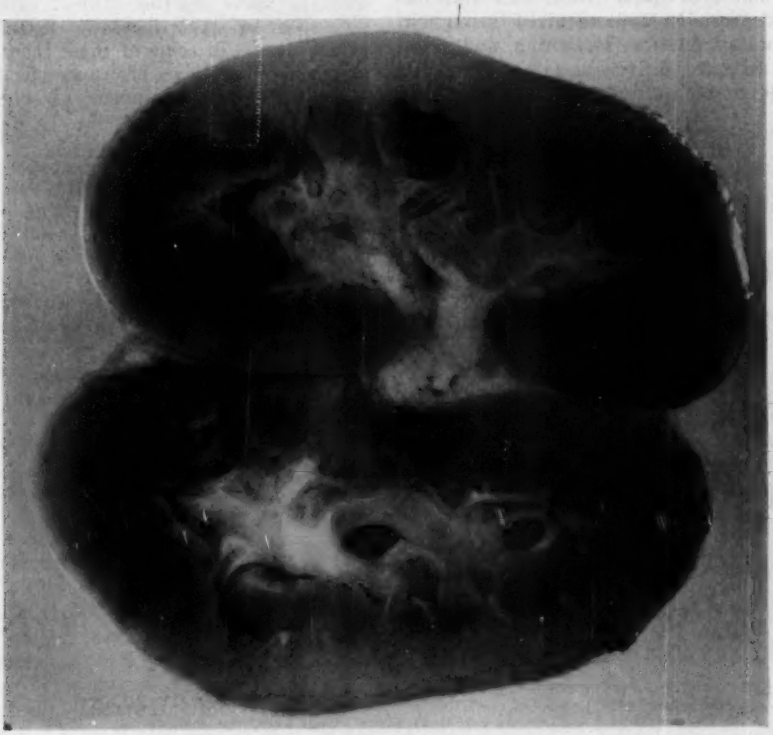

Fig. 7—Kidney from normal pig (H19300), free of urates. x 2.6.

tendency for *alpha* cells to be located in close proximity to capillaries, while *beta* cells are found in the less vascular areas of the islets (fig. 10 and 11).

DISCUSSION

Results of pathological studies, while not shedding much light on the pathogenesis of hypoglycemia, supported the chemical findings[11] in that they actually confirmed some of the chemical results and failed to provide any other basis of interpreting the syndrome than that which had been suggested by Sampson *et al.*[14] While histopathological techniques are most useful in determining many facts about tissues and tissue processes, their limitations in certain fields became evident in this study. For example, the determination of hepatic glycogen content by histopathological methods was only roughly quantitative at best. This agrees with the observations of Grafflin, Marble, and Smith.[5]

For fine cytological detail, such uniformity and precision were required throughout processes of fixation, sectioning, and staining as to make entirely satisfactory results difficult to obtain. This was particularly true regarding the pancreas in that, even when pieces of tissue 1 to 2 mm. in thickness were placed in fixative, considerable variation was noted between staining properties of the innermost part of the tissue and that first reached by the fixative. Variations in staining resulted which interfered considerably with interpretations involving the nature of secretory granules within the cells. Another variable difficult to control in the case of the porcine pancreas was thickness of sections. An effort was made to obtain sections no thicker than 4 μ; however, the brittle character of the tissue frequently precluded that possibility. In future studies, it would probably be advisable to employ some other method of dehydration in an effort to reduce brittleness and improve sectioning properties of the tissue.

While fasted pigs and clinical cases showed marked similarities, some minor variations were observed. The significance of fatty changes seen in livers and kidneys of some of the clinical cases, and not seen in experimentally fasted pigs, is not clear. In some of these cases, hypoglycemia may have been complicated by other factors. Development of acidosis or

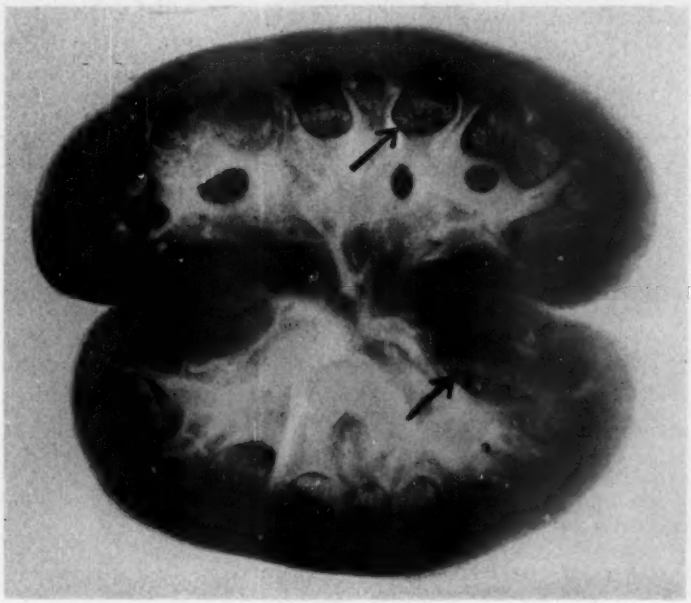

Fig. 8—Kidney from fasted pig (H19303), showing urates in the papillary ducts. x 2.8.

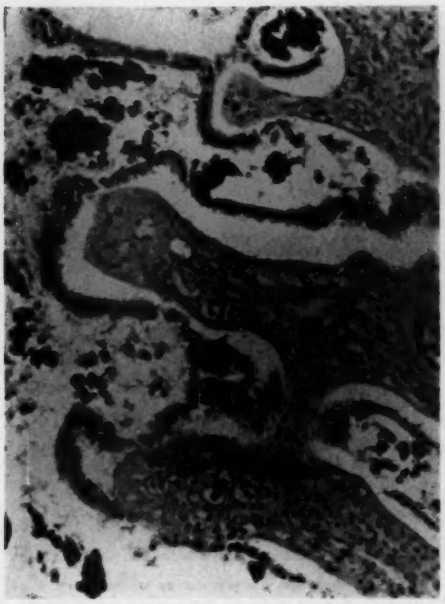

Fig. 9—Section through renal papilla of pig with naturally acquired hypoglycemia (H102953) showing urates in the papillary ducts and pelvis. Schultz-Schmidt. x 150.

ketosis might easily bring about such fatty changes, as has been noted in older animals of this and other species, particularly in sheep.[13] The possibility of the presence of some other toxic substance has not been completely eliminated. More extensive analysis of blood from clinical cases for the presence of acidosis, free phenols, guanidine, or other possible endogenous toxic substances appears desirable. Lack of regularity in occurrence, distribution, and extent of fatty changes in the clinical cases suggests that they may be of secondary concern; or that they may have resulted ultimately from some variable factor such as degree of deprivation of food or the time element, occurring in pigs which have survived for a longer period as a result of having obtained some food.

The finding of multiple petechial hemorrhages on the surface of the kidney cortex in normal pigs under 3 to 4 weeks of age has been noted by the writer for some sixteen years. Apparently this feature has escaped attention of textbook authors. Since this macroscopic finding in an older animal would have considerable pathological significance, it is obvious that the pathologist to whose attention the fact has not been drawn might easily make erroneous deductions when confronted with the "lesion". Histopathological examination of this series of baby pigs has shown rather clearly that at birth the kidney is not fully developed; the peripheral zone of the cortex contains numerous cells from which the outermost nephrons, at least, have not yet been formed (fig. 12). The histological picture suggests that as blood begins to flow into the newly formed glomeruli or other vessels during the histogenesis, it may be pushed beyond the completely formed channels into an imperfect portion with the result that it escapes the vessels, the escape constituting hemorrhage (fig. 13).

The nature of the eosinophilic inclusions in the epithelium of the jejuno-ileum of control pigs and 4 newborn pigs which had suckled is not known, but it probably repsents some fraction of the chyle which had been absorbed by the epithelium and was on its way to the point of entrance into the

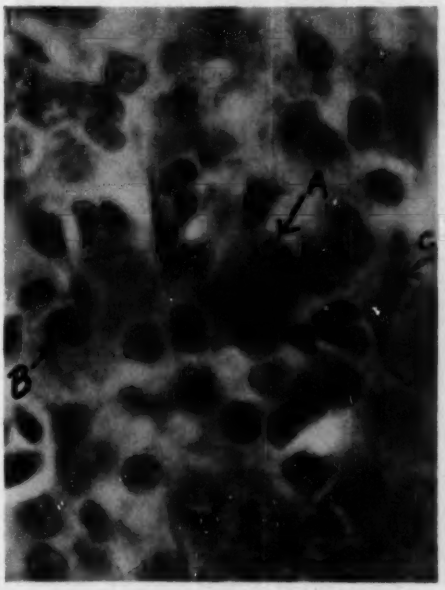

Fig. 10—Section through pancreatic islet of a newborn pig (H35361). Alpha cells (A) are shown in proximity to capillary endothelial cells (C), while the beta cells (B) occupy the less vascular parts of the islets. Chrome hematoxylin-phloxine. x 1,200.

lacteals or capillaries when the animal was killed and the tissue fixed. It may even represent unchanged protein.[10]

The significance, if any, of what appears to be a slightly greater proportion of *alpha* cells in the hypophyses of suckled pigs than in other groups is not clear. Nelson[12] reported that in the pig embryo the *beta* cells appear at a stage in which the growth- promoting hormone is first demonstrable, while the *alpha* cells are not numerous until a much later time, showing a marked increase at the time the gonad-stimulating hormone is first demonstrable. On the other hand, it is recognized that in man, at least, the *alpha* cells are almost certainly responsible for the growth hormone.[2] Certainly, the suckled pigs in this study were showing evidence of growth; in fact, there appears no reason to doubt the capacity for normal growth in the fasted pigs or clinical cases. Furthermore, any attempt to correlate the apparent finding of this study with a lack of ability to mobilze the body fat and protein stores, as might be suggested by the report of Samuels, Reinecke, and Ball,[15] must assume that lack to be universal in newborn pigs.

Fig. 11—Section through pancreatic islet of a newborn pig (H35361). Alpha cells (A) are shown in proximity to capillary endothelial cells (C), while the beta cells (B) occupy the less vascular parts of the islets. Chrome hematoxylin-phloxine. x 1,200.

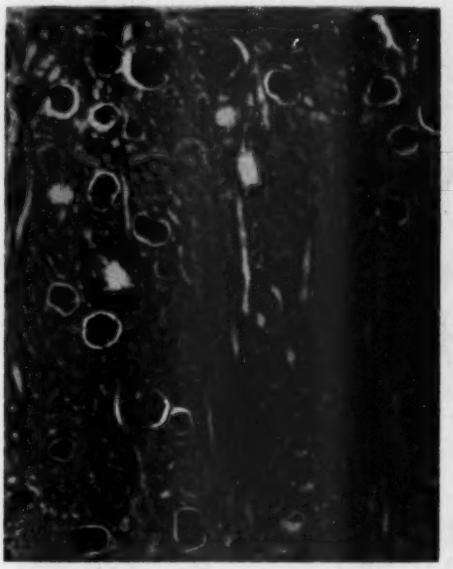

Fig. 12—Section from kidney cortex of newborn pig (H103655) showing state of incomplete development of peripheral portion. Hematoxylin-eosin. x 90.

Further investigation of a number of aspects of the problem of hypoglycemia seems desirable. For example, additional data are needed on the effects of dehydration in fasting pigs, and on the concentrations of blood-free phenols, guanidines, or other possible toxic substances in comatose pigs. From the practical standpoint, it would seem desirable to determine, if possible, that degree of hypoglycemia at which irreparable damage to the central nervous system is likely to occur,[1, 16, 9] and whether a decline in body temperature or other readily measurable effect can be used as a fairly reliable guide in treatment and prognosis. Refinement of histopathological technique and a study of greater cytological detail in the pancreatic islet cells appear desirable in order to determine the identity and ratio (proportions) of various cell types, exhaustion of secretory granules, and the possible presence of degenerative changes. Another, and perhaps more satisfactory, approach to the problem of detecting possible hyperinsulinism would be the injection of blood from pigs in hypoglycemic coma into hypophysectomized-depan-

creatized animals.[7] Further studies to round out the experimental observations also might well include determination of basal metabolic rates and quantitative studies on the urine in both normal and fasted pigs and in naturally occurring cases of hypoglycemia. In field outbreaks in which it is apparent that the pigs have suckled, but have hypoglycemia in spite of the presence of milk in the stomach, additional tests such as oral and intravenous glucose tolerance tests might be used to check on absorption of sugar. In view of the possibility of a failure of normal gastric and intestinal motility in these cases, the possible effect of environmental temperature on peristalsis and the use of lentin, arecoline, or some other drug which gives the effect of parasympathetic stimulation might be investigated. It might also prove fruitful to study the relation of the composition of sows' milk and gastric emptying time, with special reference to fat content of the milk.

SUMMARY AND CONCLUSIONS

Morphological observations on tissues selected from 147 baby pigs are reported. Included are 29 apparently normal newborn pigs killed for study between one and fifteen hours after birth, 40 apparently normal pigs allowed to suckle their dams over a period of fifteen to 150 hours, 55 pigs fasted until moribund, and 23 pigs affected with a naturally occurring syndrome in which hypoglycemia was a prominent feature. Morphological observations on liver, kidney, stomach, duodenum, jejuno-ileum, pancreas, adrenal, thyroid, cerebrum, and hypophysis are recorded.

Results of histopathological examinations, as carried out in this study, furnish support for the hypothesis that the experimental syndrome observed is due primarily to hypoglycemia. This support is chiefly embodied in the histological demonstration of depletion of hepatic glycogen and the lack of evidence of significant urinary obstruction in the kidneys of fasted pigs. These findings, when considered in the light of observation on human infants and the additional fact that apparently normal micturition was frequently observed in fasted pigs, suggest that there was no signficant disturbance of kidney function, and that the observed increase in concentration of blood nonprotein-nitrogenous compounds represents a prerenal or extrarenal azotemia, associated with protein catabolism and dehydration rather than true uremia.

The striking resemblance of certain cases of naturally occurring hypoglycemia of newborn pigs to cases of hypoglycemia induced by fasting suggests that the naturally acquired disease may be due to either a lack of nutrients or to an inability to use ingested nutrients because of a disturbance of normal digestive processes. However, the possibility of the existence of some complicating factor or factors is suggested in certain cases in which duration of the syndrome is extended by the histopathological demonstration of fatty metamor-

TABLE I—Occurrence of Urate Crystals in Kidneys of 29 Newborn, 40 Suckled, and 55 Fasted Pigs and 23 Clinical Cases

Crystals	Newborn pigs	Suckled pigs	Fasted pigs	Clinical cases
—	29	37	24	11
±	0	3	19	4
+	0	0	8	2
++	0	0	2	2
+++	0	0	2	3
++++	0	0	0	1

— = negative for crystals; ± = up to four or five tubules on the cut surface contain crystals; + to ++++ = varying amounts of urates from a few flakes in each papilla to an abundant orange precipitate in papillary ducts and pelvis.

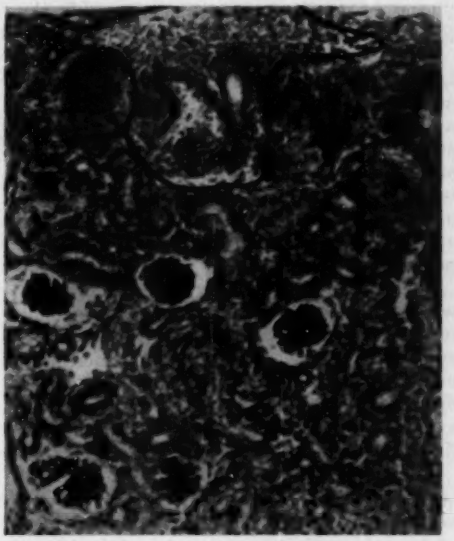

Fig. 13—Section of peripheral kidney cortex of newborn pig (H103655) showing focal hemorrhage (outlined) which appeared macroscopically as a petechia. Hematoxylin-eosin. x 225.

phoses in varying degree and extent in the livers and kidneys.

Histopathological determination of certain substances such as glycogen and uric acid in tissues is only roughly quantitative and should not be relied upon to demonstrate lesser concentrations of such substances. Furthermore, for adequate demonstration of cytological detail in the islets of Langerhans, sections of porcine pancreas should be less than 4 μ in thickness and perhaps as thin as 2 μ.

The kidney of the baby pig up to 3 or 4 weeks of age may, and usually does, present numerous fine petechial hemorrhages on its cortical surface. Since these hemorrhages evidently occur normally in postnatal development of the porcine kidney, care must be exercised in the interpretation of such a finding in pigs within this age range.

Further research is needed for the full understanding of etiological factors involved in certain, naturally occurring cases of hypoglycemia in young pigs. Use of the nonspecific term, "baby pig disease," should be avoided whenever possible, particularly when the etiological factors are known.

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Toxoplasmosis
I. Toxoplasma Isolated from Swine

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TOXOPLASMA IS known to cause disease in many species of animals including man; however, no report of toxoplasmosis in swine has been found. The isolation and identification of Toxoplasma in serious, recurring outbreaks of disease in Ohio swine extends the host range of this protozoon to a new species.

LITERATURE REVIEW

Toxoplasma infection has been described in the following animals: rabbit in 1908,[20] gondi in 1909,[12] dog in 1910,[11] mole in 1910,[15] domestic pigeon in 1911,[1] mouse in 1913,[18] rat in 1914,[10] squirrel in 1914,[4] monkey in 1916,[23] guinea pig in 1916,[2] snake in 1916,[14] lizard[5] and wombat[6] in 1932, baboon in 1933,[19] vole in 1935,[7] canary in 1937,[21] chimpanzee in 1938,[9] man in 1939,[25] sheep and cat in 1942,[13] hare in 1948,[3] chinchilla in 1949,[8] and kelpie in 1950.[22] A host of wild birds too numerous to mention have been reported to be infected with Toxoplasma without acceptable proof as to the identity of the protozoon.

MATERIALS AND METHODS

Eleven sick hogs representing various ages from 1-day-old pigs to adults over 1 year old were brought to the pathology laboratory for study. These hogs came from a Madison County, Ohio, farm where a recurring undiagnosed disease existed in swine for many years and had been extremely serious since 1949. All ages were affected, from 1-day-old pigs to adults 1 year old; however, losses were greater among the young pigs. Healthy hogs purchased from a disease-free farm became sick when placed on the problem farm. Presently known therapeutic and prophylactic measures (including hog cholera antiserum) failed to alleviate this undiagnosed disease.

Eight animals representing various stages of disease were destroyed, necropsies performed, and tissues collected for histopathological study. Necropsies were performed shortly after death on 3 additional pigs which had been held for observation during the entire course of their illness. Representative tissues were fixed in formol-saline, embedded in paraffin, sectioned at 6 μ, and stained with Delafield's hematoxylin and eosin. Tissues were also collected for bacteriological examination and animal inoculation. Brain, heart, lung, liver, spleen, mesenteric lymph node, and kidney tissues were collected aseptically from each of the 8 pigs which were killed. Emulsions of each organ (except for pooled liver and spleen) from individual pigs were prepared using approximately 1 to 2 Gm. of each organ and 5 ml. of isotonic sterile saline. Each organ emulsion was inoculated intraperitoneally into 2 white Swiss mice using 0.5 ml. per animal.

Serological studies were conducted according to the method of Sabin and Feldman,[17] who described dyes as microchemical indicators of a new immunity phenomenon affecting Toxoplasma. Toxoplasma organisms of canine origin isolated at this laboratory, as well as human Toxoplasma, were used in the comparative and serological studies.

Two healthy pigs, one of which had been proved to be immune to hog cholera by previous challenge with virulent hog cholera virus, 2 healthy dogs, and 87 white Swiss mice were used in pathogenicity studies.

RESULTS

The presence of Toxoplasma was not suspected until a microscopic study of tissues from a gilt which had died of the disease revealed round, oval, or crescentic basophilic bodies in the lesions. The similarity of these bodies to those seen in toxoplasmosis of other species caused us to suspect that they were Toxoplasma. Organisms were demonstrated microscopically in the tissues of 2 additional pigs which died and in 5 on which euthanasia was performed in various stages of the disease.

Morphology of Toxoplasma in Tissue Sections.—Under the oil-immersion objective (x 950), the organisms were found

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The assistance of Dr. R. H. Packer in the field studies, and the advice of Dr. Albert B. Sabin is greatly appreciated. This investigation was conducted in coöperation with the Division of Animal Industry, Ohio Department of Agriculture.

extracellularly as single bodies or as groups within cells. One to 8 parasites were seen within monocytes located in the lesions. Prominent lesions included pneumonia, lymphadenitis, ulcerative and fibrinonecrotic colitis, and hepatitis. The organisms measured 1.4 to 1.8 μ wide and 1.4 to 3.6 μ long and stained deeply basophilic with hematoxylin. This deep blue staining was dense at the periphery of the protozoon, while the central parts of many were lightly or incompletely stained. Round, oval, and crescentic forms were most common. Some were elongated and falciform, occurring intimately in pairs suggesting longitudinal fission. Many of these pairs were attached at their poles but separated at their midportion in various stages of longitudinal division. An occasional spherical protozoon was acidophilic except for a small, eccentric, falciform, basophilic, chromatin body. Another form was similar except that the chromatin body occupied most of the cell.

Isolation of Toxoplasma by Animal Inoculation.—Realizing that definitive identification of Toxoplasma can not safely be made only on the basis of their morphology in histological sections, mouse inoculations were performed in an attempt to recover the protozoon. Separate emulsions of aseptically collected brain, heart, lung, pooled liver and spleen, mesenteric lymph node, and kidney tissues from each of 8 affected pigs were prepared in sterile, isotonic saline. An emulsion of each organ from individual pigs was inoculated intraperitoneally into 2 white Swiss mice using 0.5 ml. per animal. In this manner, each organ, except for pooled liver and spleen of each pig, was tested for the presence of Toxoplasma by mouse inoculation. Within fifteen days after primary inoculation, 2 mice which had received a pooled liver and spleen emulsion manifested distended abdomens, incoördination, and tremors. By paracentesis, 0.5 to 1.5 ml. of translucent peritoneal exudate was obtained. Wright's stained smears made with this exudate contained organisms morphologically identical to Toxoplasma.

Another primary isolation of Toxoplasma was made in mice from the heart of a second pig. This organism had been serially passed through mice, a phenomenon which is characteristic of Toxoplasma isolated from other species.

Morphologically, in Wright's stained smears of mouse peritoneal exudate, the organisms were pleomorphic but most often lunate, pyriform or ovoid in outline, having a distinct cytoplasmic-limiting membrane that gave the impression of being stippled. They measured 1.9 to 2.2 μ in width by 3.7 to 5.7 μ long (fig. 1). The abundant cytoplasm contained basophilic granules of uneven sizes separated by clear unstained cytoplasm. No appendages or accessory structures were noted. One pole of the parasite was sharply pointed, while the opposite pole was relatively rounded. A purplish red-staining chromatin body was seen which usually occupied the rounded end. In many organisms, this chromatin body was not discernible and, in a few instances, it was central in position (fig. 2 and 3). Many parasites appeared to be in stages of longitudinal fission.

When stained with alkaline-buffered-methylene blue and prepared as a wet mount in isotonic saline by the method of Sabin and Feldman,[17] motility was not observed. However, when seen in fresh unstained wet mounts, an occasional protozoon manifested a sluggish, undulating movement of its entire body. The morphology of this organism isolated from swine conformed with the description of the "R.H." strain of Toxoplasma isolated from a 6-year-old boy by Sabin.[16] Likewise, it is morphologically identical to a canine strain of Toxoplasma and a human strain maintained by this laboratory.

Fig. 1—Toxoplasma isolated from swine. Smear of mouse peritoneal exudate showing 8 crescentic organisms. Wright's stain. Photomicrograph x 1125.

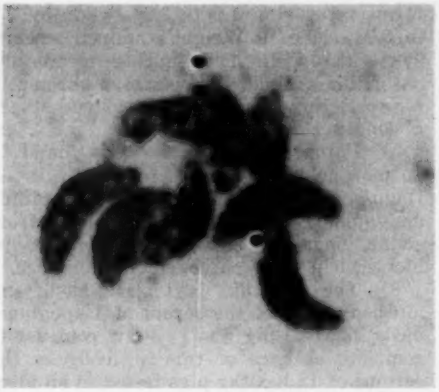

Fig. 2—Highly magnified Toxoplasma of swine showing crescentic form, granularity of cytoplasm, stippled-appearing limiting membrane, and eccentrically positioned chromatin body. Wright's stain. Approximately x 4500.

Serology.—Further attempts to identify the parasite as Toxoplasma included use of the swine organism as the antigen in the cytoplasm-modifying test described by Sabin and Feldman.[17] Using serum known to contain antibodies against human Toxoplasma, the swine organism manifested a definite cytoplasm-modifying phenomenon identical to that of the human organism which had been previously used in the original titration of the serum. A positive serological reaction also occurred when the porcine Toxoplasma was subjected to serum containing antibodies against canine Toxoplasma. Normal human serum and saline controls were negative for cytoplasm-modifying characteristics of the protozoon.

These serological studies show that the organism isolated from swine is antigenically similar to the Toxoplasma of human origin as well as to Toxoplasma of canine origin.

In further tests, serum of a pig with spontaneous disease, previously heated to 56 C. for thirty minutes to destroy natural anti-Toxoplasma factors present in animal serums, was added to human Toxoplasma, suspended in human serum, and incubated at 37 C. for one hour. In wet mounts treated with alkaline-buffered-methylene blue, definite modification of the cytoplasm occurred in 82 per cent of the organisms at a serum dilution of 1 : 40. Simultaneously, all serums from 12 healthy pigs from 6 weeks to over 1 year of age tested in an identical manner failed to modify the cytoplasm of human Toxoplasma when undiluted or in all dilutions through and including 1 : 160. Identical results were obtained when the canine Toxoplasma organism was substituted for the human strain in the test. Saline as well as normal human serum controls were negative. This further demonstrates the antigenic similarity of porcine Toxoplasma to both human and canine Toxoplasma.

One healthy pig (purchased from a farm without known disease problems), whose serum contained no demonstrable cytoplasm-modifying antibodies, was inoculated with the mouse tissues containing Toxoplasma of swine origin. Thirty days later, serum from this pig possessed a titer end point of 1 : 160 and Toxoplasma organisms were recovered from the pig by mouse inoculation.

Pathogenicity.—No significant etiological agent other than Toxoplasma was found in any of the 11 hogs studied, although attempts were made to incriminate the known infectious diseases of swine by cultural, histopathological, and animal inoculation studies. All hogs on the farm had been immunized with hog cholera serum and virus, except baby pigs nursing immune sows.

Two healthy pigs, 2 dogs, and 87 mice were inoculated with porcine Toxoplasma. The organism was found to be pathogenic

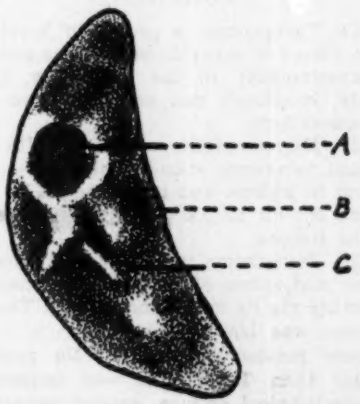

Fig. 3—Drawing of a porcine Toxoplasma as seen in Wright's stained mouse peritoneal exudate. x 13,500. A = chromatin body; B = limiting membrane; C = chromatin network.

for pigs, dogs, and mice. Tissues from these animals with artificially induced toxoplasmosis were emulsified, inoculated into mice, and again Toxoplasma organisms were recovered.

DISCUSSION

It is generally believed that toxoplasmosis of all animal species is caused by a single species of Toxoplasma, since this protozoon isolated from various animals is morphologically, antigenically, and pathogenically similar. Some previous investigators who have extended the host range have given the parasite a specific name, according to the host species from which it was isolated. However, since all Toxoplasma organisms thus far studied are alike, there is no sound basis for assigning different species names to organisms recovered from different animals. Therefore, a satisfactory designation for Toxoplasma isolated up to this time from animals, including swine, is *Toxoplasma gondii*, the original binomial name given to the organism.[12]

Not only was this porcine strain of the organism found to be morphologically and serologically identical to two known strains of Toxoplasma, but it also proved to be pathogenic for swine, dogs, and mice. The parasite isolated from swine, therefore, manifested an absence of host specificity which is another characteristic of the genus Toxoplasma.

CONCLUSIONS

1) Toxoplasma, a protozoon heretofore not known to occur in swine, was observed microscopically in the pulmonary, lymph node, intestinal, and hepatic lesions of 8 diseased hogs.

2) Microscopically, the parasite was found to assume round, oval, or crescentic form in lesions, and measured 1.4 to 1.8 μ wide by 1.4 to 3.6 μ long in sections of fixed tissues.

3) Toxoplasma was recovered from the liver and spleen of 1 pig and the heart of another pig by mouse inoculation. The protozoon was identified in Wright's stained mouse peritoneal exudate. No pathogen other than Toxoplasma was isolated by bacteriological culture, animal inoculation, or mycological culture.

4) By animal inoculation, the organisms were recovered in lunate or ovoid forms measuring 1.9 to 2.2 μ wide by 3.7 to 5.7 μ long in Wright's stained smears. They had a distinct limiting membrane, basophilic granular cytoplasm, a chromatin network, and were seen in various stages of longitudinal fission.

5) Porcine Toxoplasma were found to be morphologically and serologically indistinguishable from Toxoplasma of canine and human origin. These organisms from swine, dogs, and man were found to be identical in their pathogenicity for mice.

6) The specific cytoplasm-modifying antibody titer of the serum of 1 spontaneously diseased pig was 1 : 40, in contrast to complete absence of this antibody in the serums of 12 healthy pigs tested in an identical manner.

7) The serum of 1 healthy pig (purchased from a farm without known disease problems) possessed no demonstrable antibodies, as measured by the Sabin and Feldman serological test.[17] A 1 : 160 titer developed in the serum of this animal thirty days following inoculation of porcine Toxoplasma.

8) Porcine Toxoplasma proved to be pathogenic for swine, dogs, and mice and was reobtained from these species in the absence of other pathogens.

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Effect of Aureomycin on Diarrhea and on the Vitamin B_{12} and Methionine Needs of the Pig

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CUNHA ET AL.[1] showed that the addition of an aureomycin-B_{12} feed supplement (Lederle) to a corn-peanut meal ration relieved the need for methionine supplementation. Jukes *et al.*[2] reported that pigs fed a corn-peanut meal ration supplemented with crystalline aureomycin grew at a considerably faster rate than pigs fed the basal ration alone. This work was undertaken to help clarify the role of aureomycin as it relates to the needs of the pig for methionine and vitamin B_{12}.

MATERIALS AND METHODS

The basal ration consisted of ground yellow corn, 56.5 lb., peanut meal (hydraulic), 41.5 lb., bone meal, 0.5 lb., limestone, 1.0 lb., and a salt-trace mineral mixture, as reported by Cunha *et al.*,[3] 0.53 lb. Vitamins A and D and the seven B-complex vitamins (thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, choline, and folic acid) were added at the levels previously described by Cunha *et al.*[1] Four purebred weanling pigs, equated with respect to breed, sex, weight, and previous dietary history, were allotted to each lot. The pigs were maintained on concrete floors which were cleaned and washed daily. The trial was conducted for seventy-seven days.

RESULTS AND DISCUSSION

Data obtained in this trial (table 1) show that the addition of either vitamin B_{12} or methionine to the corn-peanut meal basal ration increased the growth rate of weanling pigs. However, when methionine was supplied in addition to B_{12} in lot 4, there was no significant difference in rate of gain from that obtained when methionine was fed as the only supplement in lot 3. This indicates that B_{12} alleviated the need for methionine on the corn-peanut meal ration employed. These findings are in agreement with previous data by Cunha *et al.*[1] with the pig.

In lot 5, the inclusion of aureomycin in the basal ration elicited a marked growth response. The addition of aureomycin plus B_{12} in lot 6 resulted in an increase in growth rate over that obtained with aureomycin alone. These data show that B_{12} is needed by the pig in addition to aureomycin if maximum growth response is to be obtained on the basal ration used. The feeding of methionine in addition to aureomycin in lot 7 was of little or no apparent benefit. Therefore, on the basis of the data obtained in this trial, it is difficult to say whether or not aureomycin decreases the methionine needs of the pig.

The level of aureomycin used in this trial (12.5 mg./lb. of feed) was considerably less than that used in earlier trials by Edwards *et al.*[4] However, this lower level of aureomycin supplementation gave comparable results in rate of gain with those obtained with the level of 22.7 mg. per pound of feed reported previously.[4] It is evident that 12.5 mg. of aureomycin per pound of feed is adequate to give a signifi-

TABLE 1—Effect on the Growth Rate of Weanling Pigs from the Addition of Vitamin B_{12}, Methionine, or Aureomycin to the Basal Ration

Lot No.	Supplement / lb of basal diet	Ave. initial wt. (lb.)	Ave. final wt. (lb.)	Ave. daily gain (lb.)
1	None	32.1	98.4	.86
2	20 μg. of vitamin B_{12} (as concentrate)*	31.5	112.8	1.05
3	1.36 Gm. of DL-methionine**	32.0	110.8	1.02
4	20 μg. of vitamin B_{12}** (as concentrate) plus 1.36 Gm. of DL-methionine	31.6	107.8	.99
5	12.5 mg. of aureomycin HCl**	31.5	121.5	1.29
6	20 μg. of vitamin B_{12} (as concentrate) plus 12.5 mg. of aureomycin HCl	32.0	146.0	1.48
7	12.5 mg. of aureomycin HCl plus 1.36 Gm. of DL-methionine	31.5	136.8	1.37

*Vitamin B_{12} concentrate which contained approximately equal parts of vitamin B_{12} and B_{13}.

**The vitamin B_{12}, aureomycin, and methionine were supplied through the courtesy of Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y.

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cant increase in growth response with the pig on a corn-peanut meal ration.

A diarrhea, which was characterized by soft, pasty feces, was observed to occur periodically throughout the trial in pigs in lots 1, 2, 3, and 4. The feces in lots 5, 6, and 7, where aureomycin was fed, were of normal consistency. These observations confirm a previous report by Jukes *et al.*,[2] in which it was found that aureomycin prevented a diarrhea on a similar ration. The pigs in lots 5, 6, and 7 also exhibited more bloom and smoothness of hair coat than the pigs in the other lots.

SUMMARY

The inclusion of either vitamin B_{12} or methionine in the diet increased the rate of gain with pigs fed a corn-peanut meal ration. No further increase in rate of gain was observed when both supplements were included in the diet. Aureomycin supplementation of the basal ration was of considerable benefit. Pigs fed aureomycin plus B_{12} gained at a faster rate than when fed aureomycin alone, thus indicating that both are needed for maximum growth response. Aureomycin prevented a diarrhea which occurred periodically in other pigs on experiment.

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Experimental Arthritis in Swine Following Multiple Injections with Erysipelothrix Rhusiopathiae

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THE ETIOLOGY of arthritis in swine is a matter of dispute. Collins and Goldie[2] carefully reviewed the early literature on the subject and the roles played by Corynebacterium, Brucella, and unidentified organisms, as well as by *Erysipelothrix rhusiopathiae*. They presented experimental evidence favoring *Ery. rhusiopathiae* as a causative agent of polyarthritis of swine. Doyle[3] summarized the evidence against *Ery. rhusiopathiae* being of etiological significance. More recently, McNutt, Leith, and Underjberg[5] reported the isolation of a nonbacterial, infectious agent which produced arthritic lesions in hogs indistinguishable from those attributed to *Ery. rhusiopathiae*.

Collins and Goldie[2] noted clinical similarities between the disease in swine, both natural and artificially induced, and rheumatoid arthritis in man. They also found microscopic and macroscopic similarities in joint pathology. Doyle[3] also observed common characteristics in the disease of man and swine. In view of the suggestion that rheumatoid arthritis in many may be due to changes in connective tissue resulting from the action of streptococcal hyaluronidase, the observation[7] that strains of *Ery. rhusiopathiae* produced hyaluronidase is interesting. However, before we could investigate the role of the hyaluronidase-hyaluronic acid system in the rheumatic process in swine, it was necessary to determine the ability of strains of *Ery. rhusiopathiae* to produce experimental arthritis consistently. This report, therefore, deals with the experimental producton of arthritis in hogs.

MATERIALS AND METHODS

Animals.—Sixteen pigs from two litters with the same sire, but different Hampshire sows, were used. Both litters were approximately the same age, having just been weaned. They were purchased from a herd in which there were no reported cases of swine erysipelas or polyarthritis. The pigs were vaccinated against hog cholera and all 10 males were castrated.

The pigs were separated into two groups, each of which contained pigs from both sows. The group which was given bacterial cultures was kept at the Animal Disease Laboratories at Reynoldsburg, Ohio. These had no outside run but were housed in a building with a cement floor which was kept bedded with dry straw. The 8 animals which were inoculated with filtrates were kept on the university grounds and were provided with an outdoor run as well as an indoor pen with a cement floor. The run was free of vegetation.

Ration.—Both groups of animals were kept on the same ration which consisted of: shelled corn, ground, 50 per cent; oats, ground, 25 per cent; soybean, ground, 14 per cent; meat scraps, 6.5 per cent; alfalfa, ground, 4 per cent; sodium chloride, 0.5 per cent. They were adequately fed but were not allowed access to more feed than was necessary for normal growth.

Bacterial Cultures and Filtrates.—Forty-eight-hour cultures of three strains of *Ery. rhusiopathiae* were used. Strain 98 had been isolated from a sow with proliferative valvular endocarditis, strain 238 from the joint fluids of a hog with erysipelas, while the history of strain 318, other than its porcine origin, is unknown. These organisms had been maintained on artificial mediums for several years. All strains produced hyaluronidase *in vitro*.

Two types of cultures were used. One was grown in necpeptone beef-heart infusion broth containing 0.25 per cent dextrose, and the other was grown in the same basal broth but containing 0.25 per cent glucurone in the place of dextrose. Glucurone was shown to increase the *in vitro* production of hyaluronidase by *Ery. rhusiopathiae*.[8]

Bacterial filtrates were made by passing 48-hour cultures through an ultrafine sintered glass filter. All filtrates were tested for sterility before using.

Subcutaneous injections were made into an axillary space. Intravenous injections were made into an ear vein. Representative protocols are given in table 1.

A tabulation of cultures of *Ery. rhusiopathiae* injected, as well as the type of medium used for growing the organisms, is given in table 2.

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TABLE 1—Schedule of Injections

Cultures			Filtrates		
Date	Amount	Route*	Date	Amount	Route
7/8	4 ml.	s.q.	7/13	5 ml.	s.q.
7/15	6 ml.	s.q.	7/15	6 mil.	s.q.
7/22	8 ml.	i.p.	7/18	6 ml.	i.p.
7/29	8 ml.	i.p.	7/25	20 ml.	i.p.
8/4	9 ml.	i.p.	7/27	20 ml.	i.p.
8/10	16 ml.	i.v.	8/3	20 ml.	i.p.
8/17	17 ml.	i.v.	8/5	20 ml.	i.p.
			8/8	20 ml.	i.p.
			8/10	20 ml.	i.p.
			8/12	20 ml.	i.p.
4/4	killed		10/19	killed	

*s.q. = subcutaneous; i.p. = intraperitoneal; i.v. = intravenous.

The injection of filtrates into the second group of animals followed the same pattern as shown in table 2.

Red and white blood cell counts as well as differential counts were made using standard procedures. The Wintrobe method was used for determining sedimentation rates.

RESULTS

No arthritic symptoms were observed in the animals injected with filtrates and, in the fifteenth week after the start of the experiment, they were killed. There was no macroscopic pathology in any of these animals. However, nine weeks after the experiment started and three weeks after the last injection, definite signs of arthritis were observed in animals receiving cultures. In a period of three weeks thereafter, 7 of the 8 animals showed arthritic symptoms. The lone hog showing no symptoms died early in the course of injections.

There seemed to be no difference among the three strains in their ability to cause the disease. Neither did it seem to matter whether the organisms were grown in the neopeptone beef-heart infusion broth containing dextrose nor in the broth with the *in vitro* hyaluronidase-stimulating glucuronic acid. Males and females were equally susceptible.

Temperatures of infected hogs ranged from 102 to 104.6 F. Temperatures as high as 105 to 110 F., which sometimes occur in acute swine erysipelas, were never observed. Infected animals seemed to walk on the tips of their toes when they were aroused. Usually they would assume a reclining position. Joints were swollen and sensitive to touch.

Hog 697, which was most severely affected, was destroyed seven months after the first subcutaneous injection. Its condition had become progressively worse. Periarticular tissue showed inflammation in the affected joint. The 4.5 ml. of synovial fluid appeared normal in every respect except excessive quantity. Attempted cultivation of organisms from the joint was unsuccessful. The cartilage on the proximal articular surface of the tibia showed extensive erosion (*see* fig. 1). No microscopic examination for the presence of histopathology was done. The arthritic condition of the other experimental hogs reached a peak in the fourth and fifth month but then improved with time and, when they were destroyed nine months after the start of the experiment, they showed no external or internal macroscopic pathology.

Fig. 1—Joint of hog 697 showing erosion of cartilage on the articular surface.

With the exception of eosinophils, the blood counts for the experimental hogs were comparable to Fraser's[4] recorded figures for hogs in the 2- to 7-month age group. Eosinophils reached a maximum of 27 per cent in 1 animal and were of that

TABLE 2—Strains and Types of Cultures of Erysipelothrix Rhusiopathiae Injected

Animal	Strain
693 male	238 (dextrose in basal broth)
694 male	238 (glucurone in basal broth)
695 male	318 (glucurone in basal broth)
696 female	98 (glucurone in basal broth)
697 male	98 (dextrose in basal broth)
698 female	318 (glucurone in basal broth)
699 female	318 (dextrose in basal broth)
700 male	318 (dextrose in basal broth)

order in several other animals at the height of the disease. These percentages fell as the animals became progressively better. However, the blood of hog 697 never exhibited more than 7 per cent eosinophils, even though it was most severely afflicted clinically.

In some hogs, there was a correlation between percentages of eosinophils and sedimentation rates, while in others there was no such correlation. Generally, higher sedimentation rates occurred at the height of the disease with a range of from 1 to 29 mm. in one hour.

DISCUSSION AND SUMMARY

An arthritic condition resembling the naturally occurring disease was produced in swine following multiple injections with hyaluronidase-producing *Erysipelothrix rhusiopathiae*. Our limited observations confirm similar studies by Collins and Goldie.[2]

It has been noted that horses being hyperimmunized for commercial antiserum production with living cultures of *Ery. rhusiopathiae* show arthritic symptoms.[1, 6] Since both horses and hogs have been known to react similarly following repeated massive injections of living organisms, one can speculate on the occurrence of a hypersensitive condition rather than a typical infection being established. However, it may also be significant that an arthritic condition may result following or during infection with all the known aërobic hyaluronidase-producing bacteria (Staphylococcus, Streptococcus, Corynebacterium, Erysipelothrix, and Pneumococcus).

In view of previous comparisons[2, 3] of the disease in man and swine, the association of hyaluronidase-producing organisms with polyarthritis of swine not only suggests the possibility that this enzyme may play a part in the pathology of porcine polyarthritis, but also adds support to the hypothesis that hyaluronidase may bear a causal relationship to various rheumatic diseases involving the hyaluronic acid of connective tissue.

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A Modified Cephalin-Cholesterol Flocculation Test for Horse Serums

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ALTERATIONS IN plasma protein fractions often occur in the disease state. These changes are only occasionally pathognomonic, nevertheless their study can often yield helpful information concerning the efficacy of treatment and the progress of disease. The cephalin-cholesterol flocculation test was introduced by Hanger,[1] in 1938, and has since proved to be a rather simple method of determining certain alterations in the plasma protein fractions of the human being. When sixteen apparently normal horse serums were subjected to this determination and run according to the method outlined by Hanger,[1] all sixteen were four plus positive at twenty-four and at forty-eight hours. Such an observation is consistent with the findings of other workers.[2, 3]

It has been shown by Moore[4] and his associates that γ globulin causes the flocculation of the cephalin-cholesterol mixture, and that an albumin electrophoretically separated from the serum inhibits this action. From an examination of the demonstrated electrophoretic proportions of the plasma proteins of both the human being and the horse,[5] it is noted that horse plasma normally contains more globulin and less albumin than human plasma. Theoretically, a positive cephalin-cholesterol flocculation test should therefore be the rule and not the exception with normal horse serums when run according to the method used for human serums. However, it has been found possible to modify this flocculation test and thus make it applicable to horse serums.

METHODS AND RESULTS

Twelve serum samples were selected and used for the preliminary work. Numbers 1 to 6 were considered normal, whereas No. 7 to 12 were believed to show evidence of liver derangement.

Series 1—Serum Dilution.—A ten-tube serial dilution of each serum sample was set up, employing 0.2 ml. of serum and 0.4 ml. of alkaline* 0.85 per cent saline in the first tube. Tubes 2 to 10 contained 0.3 ml. of alkaline 0.85 per cent saline. This resulted in serum dilutions ranging between 1 : 3 and 1 : 1536. The test employed 0.3 ml. of the serum-saline mixture, 1.0 ml. of the alkaline 0.85 per cent saline, and 0.1 ml. of the cephalin-cholesterol antigen prepared according to the directions of Hanger.[1] The relation of the antigen to the total material in this series was 1 : 14 and was kept constant.

The results showed that five of the six assumed normal serum samples had an end point at 1 : 96 serum dilution, whereas the six assumed pathological samples went to 1 : 192 serum dilution and higher.

Series 2 — Antigen Dilution. — The cephalin-cholesterol antigen prepared according to the directions of Hanger[1] was further diluted with alkaline 0.85 per cent saline to the following dilutions: 1 : 6, 1 : 7, 1 : 9, 1 : 10, 1 : 11, 1 : 12, 1 : 13, 1 : 15, 1 : 18, and 1 : 21. This series employed 0.02 ml. of serum, 0.1 ml. of antigen-saline mixture, and 1.0 ml. of alkaline 0.85 per cent saline. This represented a constant of 1 : 56 of serum to total material.

The results of the antigen dilution showed all twelve samples positive from 1 : 6 through 1 : 15. At 1 : 18 and 1 : 21 antigen dilutions, there was no significant difference between the six assumed normal samples and the six assumed pathological samples.

When the data from series 1 and series 2 were placed on a single chart and recalculated on the basis of serum dilution, antigen dilution, and serum-antigen relationship, it was found that a point existed in which a differentiation could be made between apparently normal horses and horses considered as showing evidence of liver derangement (*see* table 1).

Series 3.—This series was essentially a duplication of series 1, with the exception that no attempt was made to adjust the pH of the 0.85

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*Approximately, 17 ml. of N/50 NaOH was added to 500 ml. of 0.85 per cent saline so that 10 ml. of this solution was neutral to 0.55 to 0.60 ml. of 0.01 N H_2SO_4 with phenolphthalein as the indicator.[6]

per cent saline prior to use. The saline that was used had been made some weeks before and was found to have a pH of 4.8.

were substituted with 0.85 per cent saline in a quantity equal to the quantities used in the test. Serial dilutions were made from this tube.

TABLE 1—Results of Series 1 and 2, Recalculated to Serum-Antigen Relationship

Series 1 Antigen to total material, 1 : 14		Series 2 Serum to total material, 1 : 56	
Serum dilution	Serum-antigen relationship	Antigen dilution	Serum-antigen relationship
1 : 3	1 : 1	1 : 6	1 : 10
1 : 6	1 : 2	1 : 7	1 : 7.5
1 : 12	1 : 4	1 : 9	1 : 6
1 : 24	1 : 8	1 : 10	1 : 5.5
1 : 48	1 : 16	1 : 11	1 : 5
1 : 96	1 : 32	1 : 12	1 : 4.5
		1 : 13	1 : 4
1 : 192	1 : 64	1 : 15	1 : 3.5
1 : 384	1 : 128	1 : 18	1 : 3
1 : 768	1 : 256	1 : 21	1 : 2.5
1 : 1536	1 : 512		

Blocked in box of series 1—normal samples negative, pathological samples positive. Blocked in box of series 2—no significant "breakpoint" between normal and pathological samples. Other areas—all samples positive.

The results showed all twelve samples to be four plus positive in the first eight dilutions (up to a serum-antigen relationship of 1 : 128).

The criterion for reading the test for series 1, 2, and 3 was the method outlined by Hanger, and consisted of an arbitrary scale running from negative to four plus. This scale was difficult to interpret except in the case of the extreme negative and four plus positive samples: negative=no flocculation; one plus=slight flocculation with turbid supernatant; two plus=flocculation with slight turbidity of fluid; three plus=heavy flocculation with very slight turbidity; four plus=heavy and complete flocculation, clear supernatant.

Series 4.—This series was set up to evaluate the test on the basis of 135 apparently normal horses. The serum-antigen relationships used were 1 : 65, 1 : 75, 1 : 85, 1 : 95, 1 : 105, and 1 : 125. The quantities of the various fluids

TABLE 2—Serum Dilution for Series 4

No.	Serum	Saline*	ml. of serum in 0.1 ml. of serum-saline mixture
1	0.1 ml.	1.18 ml.	.00781
2	0.1 ml.	1.38 ml.	.00676
3	0.1 ml.	1.58 ml.	.00595
4	0.1 ml.	1.78 ml.	.00532
5	0.1 ml.	1.98 ml.	.00480
6	0.1 ml.	2.38 ml.	.00403

*Alkaline 0.85 per cent saline.

used were increased to insure a minimum of pipetting error. The test was run as recorded in tables 2 and 3; the results appear in table 4.

A more mechanical method of evaluation was used in series 4. This consisted of setting up standards corresponding to the same amount of antigen used in the test while the other fluids

The equivalents are as follows:[5] negative—equal to the undiluted standard; one plus=intermediate between the undiluted standard and a dilution of twofold; two plus=twofold dilution; three plus=fourfold to eightfold dilution; four plus=sixteen-fold dilution (water clear).

DISCUSSION

From the results of series 1 and 2, it is apparent that a suitable end point can be established for the cephalin-cholesterol flocculation test when horse serum is used. In series 1, five of the six normal samples became negative above 1 : 96 serum dilution, whereas all six samples from animals assumed to have liver derangement were positive at 1 : 192. An antigen dilution was tried (series 2), and it was found that no separation could be made between the apparently normal and the pathological serums. When the data was recalculated to a serum-antigen relationship, it was found that the dividing line between the six normal and the six pathological samples was close to 1 : 64.

Series 3 showed the necessity of keeping the 0.85 per cent saline at a definite pH, so that duplication of results would be assured. In a flocculation test, it is quite possible to have isoelectric point precipitation occur if the pH is below the physiological limits maintained in the living animal. Furthermore, it is necessary to adjust the pH of the saline just prior to use, if one is to be able to duplicate end points.

The usual method in vogue for evaluating a flocculation test implies an accuracy which is undeserved and difficult to dupli-

cate in the hands of different workers. If one considered a negative test that which showed no flocculation at the end of forty-eight hours and anything else as positive, alkaline 0.85 per cent saline. A known positive serum sample should be run along as a control.*

The usual precautions must be observed

TABLE 3—Test Proper for Series 4

No.	Serum-saline mixture	Antigen	Saline*	Serum dilution	Serum-antigen relationship
1	0.1 ml.	0.5 ml.	6.0 ml.	1 : 781	1 : 65
2	0.1 ml.	0.5 ml.	6.0 ml.	1 : 903	1 : 75
3	0.1 ml.	0.5 ml.	6.0 ml.	1 : 1015	1 : 85
4	0.1 ml.	0.5 ml.	6.0 ml.	1 : 1147	1 : 95
5	0.1 ml.	0.5 ml.	6.0 ml.	1 : 1269	1 : 105
6	0.1 ml.	0.5 ml.	6.0 ml.	1 : 1513	1 : 125

*Alkaline 0.85 per cent saline.

this objection would be ill founded. Ernst and Dotti[7] have pointed out that about 25 per cent of normal human serums exhibit a plus cephalin-cholesterol test, unless one considers zero to two plus as being normal. Kibrick and Clements[8] likewise considered the sample as normal through two plus flocculation. It was noted that this same relationship existed with horse serums, and that only three and four plus positive flocculations should be considered positive. The use of standards thus became a necessity in order to separate a negative test from a positive one. The standards used for series 4 were found to be quite satisfactory.

A series of 135 apparently normal horses was tested, including both Thoroughbreds and Standardbreds from several farms. The results demonstrated that a two plus or less at 1 : 85 serum-antigen relationship was negative for 98.4 per cent of these animals.

TABLE 4—Results of Series 4

Serum-antigen relationship	Less than 1 : 65	1 : 65	1 : 75	1 : 85	1 : 105
No. horses	77	55	1	1	1
Per cent of total	57.0	40.7	.74	.74	.74

It is recommended for routine work that the test be run in one tube, at a serum-antigen relationship of 1 : 85, using quantities of materials as outlined in series 4. Thus, 0.1 ml. of serum plus 1.58 ml. of alkaline 0.85 per cent saline will make the proper serum dilution. To 0.1 ml. of this serum-saline mixture, add 0.5 ml. of cephalin-cholesterol antigen and 6.0 ml. of

*A positive serum sample can be kept at icebox temperature (5 C.) and remain positive for months so long as the sample remains sterile.

to obviate the possibility of false positives. Some of these precautions are the use of clean glassware, freshly prepared antigen, sterile saline, the careful shaking of the tubes to insure an even suspension, the stoppering of the tubes with cotton plugs to keep out contamination, and lastly, the placing of the tubes in the dark as soon as possible after the addition of the antigen. It is important that a temperature of 20 to 25 C. be maintained during the developing period. After forty-eight hours, any sample that is two plus or less at this dilution is considered negative, whereas a sample that is three plus or higher is considered positive.

From data published elsewhere,[9] horses experimentally infected with equine infectious anemia** showed, on the average, a three plus positive cephalin-cholesterol flocculation test at 1 : 85 and up to 1 : 145, starting with a date just prior to the second acute attack. These animals remained positive during the acute and subacute stages. The 1 carrier animal which was available for study remained positive during the seventeen months of weekly examinations, although this animal exhibited four acute attacks during this period. Here is one disease in which a positive cephalin-cholesterol flocculation test is one of the features. In no way is the foregoing meant to imply that this test is pathognomonic for equine infectious anemia.

The mechanism of this test has been amplified by Moore[4] and his associates. They demonstrated that a positive flocculation will occur when (1) there is a rise in γ globulin, (2) albumin falls below the amount necessary to inhibit the globulin, or (3) there is a reduction in the inhibit-

**Material secured from the New Hampshire outbreak in 1947.

ing fraction of the serum albumin. Thus, flocculation may be expected to occur in primary hepatic impairment. However, flocculation may also occur in conditions in which the reticulo-endothelial system is affected without primary hepatic involvement, and the mechanism of the positive reaction is the same as in liver disease. Malaria is an example of this type.[10]

It is hoped that others investigating diseases of the horse will find this modified cephalin-cholesterol flocculation test a useful aid in diagnosis.

SUMMARY

1) A modification of the cephalin-cholesterol flocculation test applicable to horse serums is presented.

2) In a series of 135 presumably normal horses, 98.4 per cent were negative to this flocculation test when run as recommended.

3) The mechanism of the positive test is such that a positive test can be expected in primary hepatic impairment and/or in conditions in which the reticulo-endothelial system is involved without hepatic impairment.

4) A positive test was rather consistently demonstrated by a group of horses experimentally infected with equine infectious anemia.

5) It is important to stress the fact that this test should be used only as an aid in the diagnosis of liver and reticulo-endothelial system disturbance, and it is not pathognomonic for any particular disease.

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A Further Note on the Action of Phenol on the Virus of Equine Infectious Anemia

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IT IS A RECOGNIZED fact that horses that have had an acute attack of infectious anemia may make an apparent recovery but still harbor the virus for years. Such animals appear normal and may thus gain entrance to institutions preparing biological products. The danger of disseminating the disease through the use of antiserums or vaccines prepared unknowingly from carriers presents a problem of great concern.

The importance of determining the effects on the virus of chemical agents, especially phenol, which is commonly used as a preservative in the preparation of antiserums, is obvious. This problem has been studied by research workers in various parts of the world with somewhat conflicting results.

Balozet[1] considers phenol to be weakly destructive to the virus and believes the virus contained in serum to be more resistant against phenol than that in spleen tissue. He found that 0.1 per cent phenol has no effect on virulent serum in twenty-four hours and requires four months and ten days to destroy the virus, whereas the same concentration of phenol in a 10 per cent saline suspension of spleen tissue destroys the virus in sixteen days.

At a concentration of 0.5 per cent, phenol inactivation of the virus in virulent serum was reported by Gerlach[2] in thirty days, by Kral[3] and by Heiermann[4] in three months, by Opperman and Ziegler[5] in ninety days, and, when held at 14 to 15 C., by Frohner and Bierbaum[6] and Fortner[7] in ninety days. On the other hand, Semenov and Oldenborger[8] reported that virulent serum treated with 0.5 per cent phenol is still infective but attenuated after six months, but consider the virus is destroyed within ten months by a phenol concentration of 0.5 per cent.

In recent experiments Miura, Kutii, and Ueda,[9] using 0.5 per cent concentration of phenol, demonstrated that the virus present in virulent horse serum can be inactivated in at least three days, if the phenolized serum is held in an incubator at 37 C. instead of under refrigeration. For the purpose of inactivating virus present in immune serum treated with 0.5 per cent phenol, they recommend that it be held at least five days at 37 C. Joseph[10] reported inactivation of the virus in two months with a concentration of 1.5 per cent phenol. The Japanese Commission[11] found that 2.0 per cent phenol destroys the virus in one hour, whereas Balks,[12] using the same concentration, found the virus to be resistant for more than twenty-four hours. Luhrs[13] reported that 4.0 per cent phenol destroys the virus in less than one hour.

Stein, Osteen, Mott, and Shahan[14] showed that the virus contained in filtered virulent serum from acute cases experimentally infected with the New York strain of virus could be inactivated in thirty days by a concentration of 0.5 per cent phenol or 0.5 per cent phenol together with 0.5 per cent ether held at 5 C. However, the virus present in virulent serum was not inactivated in sixty days at 5 C. by either 0.25 per cent phenol together with 0.25 per cent ether, or by 0.35 per cent phenol together with 0.35 per cent ether.

ACTION OF 0.5 PER CENT PHENOL ON THE NEW HAMPSHIRE STRAIN OF VIRUS

These studies were carried out to obtain additional information on the action of phenol on the virus and to ascertain if the New Hampshire strain of virus was more resistant to phenol than the New York strain, which had been used in our previous studies.

The virulent serum used in the present experiments was obtained from an acute case of infectious anemia in horse 1485 and represents the third passage of the virus through horses since its isolation in 1947 from an outbreak in race horses.

The blood was collected aseptically in large tubes and allowed to clot. The serum was then removed for use in the experiments and divided into two lots. Lot 1 was untreated and held under refrigeration at 5 C. for use as a control. Lot 2 was placed in an Erlenmeyer flask and

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sufficient phenol was added slowly during constant agitation to give a concentration of 0.5 per cent.

Samples from lots 1 and 2, after being held under refrigeration at 5 C. for thirty and ninety-two days, were inoculated subcutaneously into normal horses to test for the presence of virus.

The results of the horse inoculation tests, which are given in detail in table 1, show that the horses that received lot 1 (the untreated samples) after thirty and ninety-two days' storage at 5 C. and the horses that received samples of lot 2 (treated with 0.5 per cent phenol and held at 5 C. for thirty days) developed symptoms of acute infectious anemia, while the horses injected with that portion of lot 2 held for ninety-two days at 5 C. failed to develop symptoms of the disease.

Pooled blood samples obtained from the horses that failed to develop symptoms of infectious anemia following injection of the 92-day-old phenolized serum of lot 2 were tested for the presence of virus and found to be negative. These horses were finally challenged with a known virulent strain of New Hampshire virus and promptly developed symptoms of acute infectious anemia, indicating that the 92-day-old phenolized serum was devoid of virus and possessed no immunizing value against the disease.

By referring to table 1, it will be noted that there is a difference in size of the inoculum injected into the various test horses.

However, in all instances except one, the amount of phenolized serum injected into test horses exceeded the amount of unphenolized serum administered to the control horses.

TABLE 1—Result of Tests to Determine the Effect of 0.5 Per Cent Phenol on the Virus of Equine Infectious Anemia Contained in Virulent Horse Serum

Material tested for presence of virus	Amount injected subcut. (cc.)	Date injected	Horse No.	Results	Challenge			
					Strain of virus	Amount injected subcut. (cc.)	Date	Results
Virulent serum New Hampshire strain 1485 treated with 0.5% phenol 30 days at 5 C.	75	11/1/50	1460	Symptoms of infectious anemia on 29th day (Subacute case)*	none	..	.....	.....
Virulent serum New Hampshire strain 1485 treated with 0.5% phenol 30 days at 5 C.	75	11/1/50	1463	Symptoms of infectious anemia on 19th day (Subacute case)*	none	..	.....	.....
Virulent serum New Hampshire strain 1485 (control)	10	11/1/50	1413	Symptoms of infectious anemia on 15th day (Subacute case)*	none	..	.....	.....
Virulent serum New Hampshire strain 1485 (control)	1	11/1/50	1221	Symptoms of infectious anemia on 19th day (Subacute case)*	none	..	.....	.....
Virulent serum New Hampshire strain 1485 treated with 0.5% phenol 92 days at 5 C.	10	1/2/51	1476	Remained well to 4/6/51 (94 days)**	New Hampshire strain 1461 oxalated whole blood	40	4/ 6/51	Symptoms of infectious anemia on 11th day (Acute fatal case)***
Virulent serum New Hampshire strain 1485 treated with 0.5% phenol 92 days at 5 C.	15	1/2/51	1471	Remained well to 4/6/51 (94 days)**	New Hampshire strain 1461 oxalated whole blood	40	.....	Symptoms of infectious anemia on 10th day (Acute fatal case)***
Virulent serum New Hampshire strain 1485 (control)	10	1/2/51	1419	Symptoms of infectious anemia on 7th day (Acute case)*	none	..	.....	.....
Virulent serum New Hampshire strain 1485 (control)	20	1/2/51	1439	Symptoms of infectious anemia on 12th day (Acute fatal case)*	none	..	.....	.....
Pooled blood from horses 1471 & 1476 94 days after receiving phenolized serum	60	4/6/51	1466	Remained well to 5/29/51 (53 days)**	Pooled blood from horses 1471 & 1476 after being infected	50	5/29/51	Symptoms of infectious anemia on 10th day (Acute case)*

*Positive for virus. **Negative for virus. ***Indicates horses were susceptible, and phenol-inactivated serum was devoid of immunizing value.

It is believed that the variation in dosage in no way affected the results of the test, since it was definitely established in previous titration tests that 1 cc. of filtered virulent serum from horses affected with the disease, when administered subcutaneously, intradermally, intravenously, or intracranially, is capable of producing clinical symptoms of infectious anemia. The same material, when injected subcutaneously in a 0.1-cc. or 0.001-cc. dose, likewise produced the disease in susceptible horses.

DISCUSSION

The results of our studies on the action of phenol on the virus of infectious anemia contained in virulent serum held under refrigeration and the conflicting results reported by other investigators who have worked on the problem indicate that the virus had considerable resistance against phenol and point to a possible variation in resistance against phenol by different strains. The investigations carried out by Balozet[1] and Miura, Kutii, and Ueda[9] indicate that temperature also plays an important part in determining the influence of phenol on the virus.

In view of the above findings, it would appear that phenolization is not a reliable method for destruction of active infectious anemia virus in immune serum derived from horses, since it is a common practice for biological institutions to store such material under refrigeration after treatment with phenol.

In previous studies,[14] we have shown that the application of heat under proper conditions is one of the most effective methods of inactivating the virus in virulent serum. This can be accomplished by heating the serum at 58 to 59 C. for one hour in a well-controlled automatic pasteurizing device, after which phenol or other chemicals may be added as a preservative.

As a safety measure to prevent the dissemination of infectious anemia, the regulations of the Bureau of Animal Industry require that all antiserums of equine origin prepared in biological establishments in the United States operating under government license must be heated at a temperature of 58 to 59 C. for one hour before any preservative is added.

SUMMARY

To determine the resistance of the virus of equine infectious anemia to the action of 0.5 per cent phenol, samples of the phenolized virulent serum, after being held thirty and ninety-two days, respectively, were injected into susceptible horses, while unphenolized samples of the same serum, held for the same period, were likewise injected into test horses as controls, with the following results.

The virus contained in the serum from an acute case of equine infectious anemia experimentally infected with the New Hampshire strain of virus was not inactivated by the action of 0.5 per cent phenol when held at 5 C. for thirty days. However, inactivation of the virus in the same lot of phenolized serum occurred when the sample was held at 5 C. for ninety-two days.

It was also demonstrated that the virus in virulent serum may remain fully active when stored at 5 C. for ninety-two days.

The inactivated 92-day-old phenolized serum, when injected into horses, was devoid of immunizing value against the disease.

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Critical Tests with Toluene for Ascarids and Bots in Horses

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THE MATERIALS in this report were obtained from a series of critical tests with toluene against ascarids (*Parascaris equorum*) in weanling and yearling horses. In the absence of an anthelmintic effective against horse ascarids when fed to infected animals in subtherapeutic amounts in their feed, this laboratory has sought an anthelmintic with ascaricidal activity superior to that possessed by carbon disulfide, *i.e.*, one more effective against immature specimens of the parasite. The most widely employed treatment in central Kentucky, carbon disulfide therapy, has shortcomings as it is now used in equine practice.[1, 2] The advantages of an ascaricide which would not lose effectiveness when administered to horses on full feed are obvious when it is considered that infections with ascarids are greater problems in sucklings, weanlings, and yearlings than in older horses. The control of ascarids in horses is still dependent upon periodic treatment at the present time.

The data on bots were compiled from the above series of horses treated with toluene.

MATERIALS AND METHODS

The majority of the horses used in the critical tests were Thoroughbreds, although a few were light-boned grade horses. All of them had naturally acquired infections with bots, ascarids, and strongyles.

The critical tests were conducted with toluene administered by stomach tube employing doses of 0.1 and 0.2 cc. per pound of body weight; the tube was flushed with a small quantity of water while still in place. All horses were on full feed when treated and were sacrificed twenty-four hours after treatment in order that worms killed and expelled from the small intestine would not disintegrate before being recorded.

At autopsy, a complete examination was made of the stomach and small and large intestines. Ascarids present were collected and counted; bots present in the above organs were also collected, counted, and identified. During the examination, the digestive and other organs were examined for gross evidence of pathological changes resulting from the treatment. The toluene used was supplied through the courtesy of Pitman-Moore Company, Indianapolis, Ind.

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EXPERIMENTAL FINDINGS

Toluene at 0.1 cc. per Pound of Body Weight.—These tests, involving 12 horses, are recorded in table 1. Of 809 ascarids present in the small intestines of the 12 horses, 473 were expelled and 383 of these were dead within twenty-four hours. The percentage efficiency of expulsion ranged from 0 to 100.0 per cent and averaged 58.5 per cent; the efficiency of toluene as an ascaricide ranged from 0 to 100.0 per cent and averaged 47.5 per cent in twenty-four hours.

Toluene at 0.2 cc. per Pound of Body Weight (Table 2).—These tests involved 8 horses, and there were 420 ascarids present in the small intestines of the horses. A total of 419 worms were expelled from the small intestines by treatment and all of these were dead when the horses were examined postmortem. At 0.2 cc. per pound, toluene's efficiency at expulsion and as an ascaricide was 99.8 per cent in twenty-four hours.

DISCUSSION

Toluene was remarkably effective against *P. equorum* both from the standpoint of expelling worms from the small intestine and as an ascaricide when administered to test horses at the rate of 0.2 cc. per pound of body weight. It was as effective against immature as against mature ascarids. It reached an efficiency of 99.8 per cent in horses on full feed. Its action against ascarids appeared to be much more rapid than that possessed by carbon disulfide. Animals treated with carbon disulfide pass ascarids in forty-eight to seventy-two hours after treatment, and the majority of specimens passed by treated animals generally are not greatly decomposed. The majority of ascarids recovered in the toluene tests were badly decomposed.

TABLE 1—Tests with Toluene at 0.1 Cubic Centimeters per Pound of Body Weight

Horse (No.)	Weight (lb.)	Amt. toluene (cc.)	Ascarids recovered: Small intestine Live	Small intestine Dead	Large intestine Live	Large intestine Dead	Kill (%)	Expulsion (%)
26	700	70	111	0	89	266	57.1	76.2
20	750	75	37	0	0	2	5.1	5.1
16	587	59	0	0	0	1	100.0	100.0
15	660	66	82	0	0	16	16.3	16.3
11	1,012	101	1	0	0	1	50.0	50.0
10	306	31	19	0	0	0	0.0	0.0
9	600	60	0	0	1	13	92.9	100.0
8	860	86	0	0	0	17	100.0	100.0
7	930	93	0	0	0	4	100.0	100.0
6	351	35	0	0	0	15	100.0	100.0
5	342	34	85	1	0	45	35.1	34.3
4	580	58	0	0	0	3	100.0	100.0
Total 12			335	1	90	383	47.5	58.5

At the rate of 0.1 cc. per pound, the action of toluene against ascarids was inferior; it had an ascaricidal efficiency of only 47.5 per cent.

Postmortem findings indicated that toluene had been well tolerated despite its irritant nature. Moderate gastritis and enteritis, characteristic of animals treated with carbon disulfide and sodium fluoride, were not in evidence. No other gross pathological changes were observed. A few animals were available for clinical observation after treatment. Five yearlings which received two treatments each with toluene at the rate of 0.1 cc. per pound exhibited no change in appearance or action. One group of 7 pregnant light-boned mares was treated, each mare receiving 200 cc. of toluene. Of this group, 1 mare foaled within four hours after treatment and the foal was normal. A second mare foaled within a week and the foal was dead at birth, having been suffocated during delivery. The other mares exhibited no after-effects of treatment and foaled normally in a six-week period. These 12 animals, however, by no means are considered to have established universal tolerance for toluene therapy at 0.1 or 0.2 cc. per pound of body weight.

In contrast to the action of sodium fluoride, toluene had an action on the two common species of horse bots in Kentucky, *Gasterophilus intestinalis* and *Gasterophilus nasalis*. Its greater and more selective action against *G. intestinalis* has been reported previously.[3] Sodium fluoride was not found to have an action against bots or strongyles;[1] toluene had no appreciable effect upon strongyles present in the test horses.

The action of toluene against bots is more rapid than the action of carbon disulfide; carbon disulfide, however, acts about equally well against both *G. intestinalis* and *G. nasalis*. For example, in the complete group of 22 horses treated with toluene, 3,304 specimens of *G. intestinalis* were recovered. Of this number, 2,666 (80.69%) were dead at postmortem examination twenty-four hours after treatment. In the same 22 horses, 909 specimens of *G. nasalis* were recovered and only 216 (23.76%) were dead twenty-four hours after treatment. The percentage of killed *G. intestinalis* specimens, when toluene was used at 0.1 or 0.2 cc. per pound of body weight, was 83.26 and 79.06, respectively. The percentage of killed *G. nasalis* specimens at 0.1 or 0.2 cc. per pound was 23.85 and 23.70, respectively.

Six mature horses were used for critical

TABLE 2—Tests with Toluene at 0.2 Cubic Centimeters per Pound of Body Weight

Horse (No.)	Weight (lb.)	Amt. toluene (cc.)	Ascarids recovered: Small intestine Live	Small intestine Dead	Large intestine Live	Large intestine Dead	Kill (%)	Expulsion (%)
22	500	100	0	0	0	26	100.0	100.0
28	620	124	0	0	0	83	100.0	100.0
29	650	130	0	0	0	6	100.0	100.0
30	600	120	1	0	0	6	85.7	85.7
31	550	110	0	0	0	22	100.0	100.0
34	660	132	0	0	0	251	100.0	100.0
35	720	144	0	0	0	15	100.0	100.0
33	700	140	0	0	0	10	100.0	100.0
Total 8			1	0	0	419	99.76	99.76

testing of carbon disulfide against bots. Each mare received 7 drams of carbon disulfide given by stomach tube. Three were destroyed at twenty-four hours and 3 at forty-eight hours after treatment. A total of 689 specimens of *G. nasalis* were recovered in the first 3 animals (those examined at 24 hours after treatment). Of this number, all were alive in the stomach and all but 4 were attached in the pyloric region. A total of 260 *G. intestinalis* organisms were recovered in the stomachs of the 3 horses; 257 specimens were alive and attached in the cardiac region, while 3 specimens were dead and were recovered in the contents of the large intestine. These figures are in contrast to 80.69 per cent dead *G. intestinalis* and 23.76 per cent dead *G. nasalis* specimens in horses treated with toluene and examined twenty-four hours after treatment. The first of the 3 horses treated with carbon disulfide was treated while on full feed. The second and third horses were fasted for twenty hours before treatment.

The second group of 3 mature horses was destroyed and examined for bots forty-eight hours after treatment. Each of these animals was fasted for twenty hours before treatment. In these animals, 69 of 74 *G. nasalis* (93.24%) and 217 of 240 *G. intestinalis* (90.42%) specimens were dead at the end of forty-eight hours. Actually, 20 dead *G. nasalis* and 24 dead *G. intestinalis* organisms were passed by the 3 horses. These percentages of dead bots were superior to those obtained from the toluene treatments but were based on fewer tests. Carbon disulfide appeared considerably more effective against *G. nasalis*.

The present series of critical tests appear to have established a specific and rapid action of toluene against horse ascarids, *P. equorum*. Toluene is effective against both immature and mature specimens of ascarids and is efficient in horses on full feed. The drug is effective against one common species of bot, *G. intestinalis*, but is not considered effective against *G. nasalis*.

Some animals showed brief periods of depression following treatment but thereafter showed no depression or inappetence. No diarrhea occurred in treated animals. No gastritis or enteritis was noted at postmortem examination. A total of 34 animals was studied and 22 of this number were used in critical testing. The tests are not considered sufficient to establish tolerance levels or to warrant use of toluene in equine therapy.

SUMMARY

A series of critical tests were conducted with toluene at the rate of 0.1 or 0.2 cc. per pound of body weight in weanling and yearling horses to determine its action against ascarids, *Parascaris equorum*, and two species of bots, *Gasterophilus intestinalis* and *Gasterophilus nasalis*.

At 0.2 cc. per pound, toluene killed and expelled 99.8 per cent of the ascarids present in the small intestines of 8 test animals in twenty-four hours. At 0.1 cc. per pound, toluene killed only 47.5 per cent of the ascarids present in 12 test animals in twenty-four hours.

In the entire series of 22 test animals, toluene killed 80.69 per cent of one bot species, *G. intestinalis*, but only 23.76 per cent of *G. nasalis* in twenty-four hours.

A comparison was made of the actions of toluene, sodium fluoride, and carbon disulfide on bots and ascarids.

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Studies on the Subgross Anatomy of the Bovine Liver, III. Comparative Arrangement of the Blood Vessels of the Livers of the Bovine and Equine Fetuses

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THE DUCTUS VENOSUS, a fetal vascular shunt by which blood of the hepatic portal vein and umbilical vein may pass directly to the posterior vena cava without passing through the liver lobules, is said to have been first described in 1564. The history of the descriptive anatomy of the vessels of the fetal liver is reviewed by Barclay, Franklin, and Prichard.[1] It is stated by these authors that the ductus venosus occurs in rodents, carnivores, ruminants, and primates. It is not found in the horse and pig fetus in the latter stages of gestation.

The presence of the ductus venosus is briefly discussed in some of the anatomy texts in current use; however, illustrations of the arrangement of the vessels of the fetal bovine and equine livers are scarce.[2] Chauveau's textbook[3] presents illustrations of the internal vessels of the fetal livers of the sheep and horse, but the keys to the illustrations are not consistent with the contents of the illustrations and text. The purpose of this paper is to augment the currently accessible veterinary anatomical literature by the presentation of an illustrated description of the arrangements of the blood vessels of the livers of bovine and equine fetuses.

METHOD OF PROCEDURE

The specimens upon which the observations of this paper were based consisted of vinylite corrosion casts of five equine fetal livers, one cast of the liver of a foal of a few days of age, and four bovine livers from fetuses or from newborn calves. A corrosion cast of the entire vascular system of an equine fetus was also utilized. The equine fetuses were estimated to be in the fourth to the eighth month of development. The calf livers were taken from fetuses at the middle of gestation or from day-old animals. In the latter cases, the umbilical veins were sufficiently patent to permit injection of plastic.

The corrosion casts were prepared in the manner reported in previous papers.[4, 5] Plastic of differing colors was injected into the hepatic portal vein, hepatic veins, and the umbilical veins. In the cases of the bovine livers, the plastic solutions injected into the three venous systems mixed. This was because of the presence of the ductus venosus, and a multi-colored cast resulted. In the cases of the equine livers, the plastic that was injected into the hepatic veins remained independent and its color was unaltered. Since the vinylite corrosion technique usually results in a complete cast (with the exception of capillaries), it was necessary to simplify the specimen by dissection to reveal the arrangement of the larger internal vessels.

RESULTS AND DISCUSSION

The accompanying figures illustrate the major observations concerning the distribution of the vessels in the fetal livers. In the horse (fig. 1), the umbilical vein passes forward in the falciform ligament, through the umbilical fissure, and enters the substance of the central lobe.* Its course in the liver substance is short. It enters the anterior ventral portion of the hepatic portal vein at the left of the portal fissure. There is no ductus venosus; all blood entering the substance of the liver by means of the umbilical vein must pass through the ramifications of the hepatic portal vein.

The umbilical vein of the calf enters the liver at the umbilical fissure. The umbilical fissure of the liver of the fetus and newborn calf is in the form of a deep channel. The umbilical vein does not give off branches for a considerable distance in its course within the liver; however, near its junction with the hepatic portal, branches are given off to the liver tissue located ventral to the portal fissure. One such branch is demonstrated in fig. 2. As in the

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*The central lobe is the terminology of Sisson. According to the description of Ellenberger and Baum,[6] the position of the umbilical fissure divides the liver into left and right lobes; therefore, the umbilical vein enters the substance of the liver between the central portions of the right and left lobes.

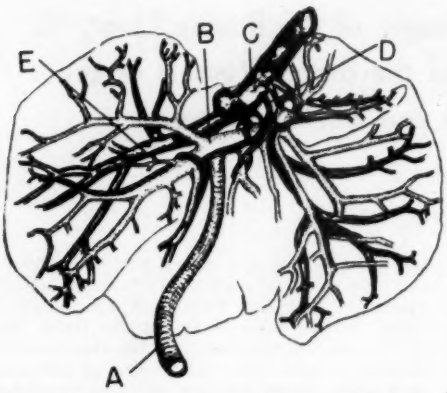

Fig. 1—Cast of the blood vessels of the liver of an equine fetus viewed from the visceral aspect. A = umbilical vein; B = hepatic portal vein; C = posterior vena cava; D = hepatic artery; E = hepatic vein.

horse, the umbilical vein unites with the portal at a point ventral and to the left of the portal fissure. The ductus venosus is a short vessel of considerable diameter which leaves the hepatic portal trunk at a point opposite and above the entrance of the umbilical vein. It arches forward, completely surrounded by liver tissue, to enter the posterior vena cava at the point of entrance of the hepatic veins from the lower portion of the liver. In viewing the intact liver from the visceral surface, the ductus venosus can be located by reference to the papillary lobe (or process), the portal fissure, and the point of exit of the posterior vena cava from the substance of the liver. It is embedded in the liver tissue just beneath the ventral border of the papillary lobe. In three of the four bovine livers, the cast of the ductus venosus was smooth and showed no branches. In one specimen, a liver from a newly born calf, the ductus venosus collected vessels from the papillary lobe and surrounding liver tissue. Since these vessels entered anterior to the position of the sphincter of the ductus venosus, it might be presumed that they would constitute a portion of the hepatic venous system after the ductus had closed.

In one of the casts from a newborn calf, a distinct constriction was noted at the origin of the ductus venosus. This probably represented the position of the sphincter muscle which was described in considerable detail in the sheep and noted in the calf by Barclay *et al.*[1]

"Hepatic portal circuits," anastomoses of various small segments of the hepatic portal venous system, were demonstrated in the vascular casts of the livers of both bovine and equine fetuses. These have been described previously in the livers of postnatal cattle.[4]

Study of a 25-cm. horse fetus showed no detectable anastomosis between the umbilical vein or hepatic portal vein, and the posterior vena cava. The vascular arrangement as found in this specimen is illustrated by fig. 3.

Illustrations of the arrangements of the vessels of the livers of the bovine and equine fetuses are to be found in foreign literature,[7, 8] but such illustrations are not to be found in accessible literature in the English language. With emphasis centralized upon a single "type" specimen in American anatomy laboratories, such

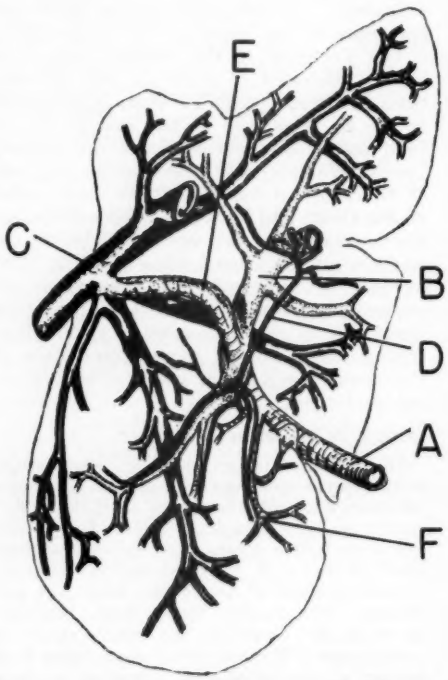

Fig. 2—Cast of the blood vessels of the liver of a fetal calf, viewed from the visceral aspect. A = umbilical vein; B = hepatic portal vein; C = posterior vena cava; D = hepatic artery; E = ductus venosus; F = branch of the umbilical vein from the substance of liver. This branch would later become a portion of the hepatic portal system.

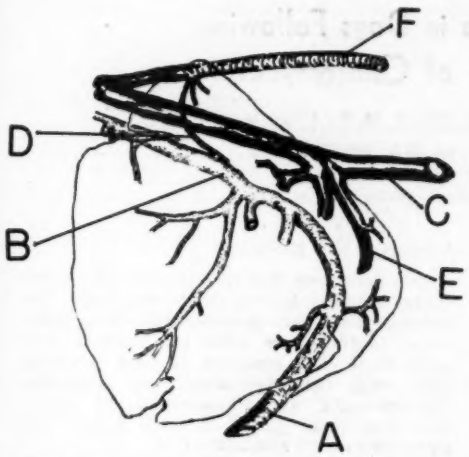

Fig. 3—Right anterior view of the major veins of the liver of a 25 cm.-equine fetus. No large connections can be seen between the portal system and the systemic venous circulation. A = umbilical vein; B = hepatic portal vein; C = posterior vena cava; D = hepatic artery; E = hepatic vein from left central lobe; F = aorta.

comparative considerations as the presence or absence of a ductus venosus in the various domestic animals is often overlooked. Without the "short-cut" afforded by the ductus venosus, all blood returning to the body of the fetus from the placenta must pass in contact with hepatic epithelial cells by means of the hepatic portal circulation. The significance of the blood flow of the umbilical and portal veins is described and discussed in considerable detail by Barclay *et al.*[1]

SUMMARY

Illustrations of the vascular arrangement of the livers of bovine and equine fetuses, as demonstrated by the vinylite-corrosion technique, are presented. Equine fetuses of four to eight months' gestation showed no evidence of anastomosis between the hepatic portal or umbilical veins, and the posterior vena cava; whereas, a well-developed ductus venosus could be demonstrated with ease in the livers of bovine fetuses and newborn calves.

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Blood Level Studies in Dogs Following the Administration of Chloromycetin

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ALTHOUGH NUMEROUS STUDIES on blood levels have been conducted following the administration of single doses of chloromycetin®* (chloramphenicol) in dogs, few data are available for multiple dose schedules that would be of practical value in setting up a therapeutic regimen.

Gruhzit *et al*.[1, 2] administered a single oral dose of 86 mg. per kilogram of body weight, obtaining blood serum-chloromycetin levels of 8 μg. per cubic centimeter at two hours, 25 μg. at four and six hours, and 20 μg. at eight hours after administration. When the dosage was increased to 150 mg. per kilogram of body weight, blood serum levels of 39 μg. were obtained two hours after the administration of chloromycetin by mouth, with 20 μg. still present after eight hours. None was detectable after twelve hours.

Dogs treated *per os* twice daily with 50 mg. of chloromycetin per kilogram had average serum levels for an eight-hour period ranging from 6.2 to 9.8 μg.; on dosage of 75 mg. per kilogram twice daily, serum levels of 19.1 to 25.8 μg. were obtained; and 100 mg. per kilogram twice daily gave average levels of 26.6 to 34.6 μg. Two hundred milligrams per kilogram twice daily showed serum levels of 80, 120, 20, and 0 at six, eight, twelve, and twenty-four hours, respectively.[2]

Intravenous chloromycetin has been used experimentally in dogs by Glazko *et al*.[3] When 50 mg. per kilogram of body weight was administered intravenously in 8 per cent propylene glycol, a rapid drop was observed in the chloromycetin-blood serum level from 38 μg. per cubic centimeter thirty minutes after injection to a nonmeasurable amount after eight hours.

Intramuscular injections of chloromycetin are not recommended in dogs at the present time.[2]

The present paper reports a systematic study of blood serum levels in dogs after oral administration of chloromycetin on various dosage schedules. On the basis of these results, a working dosage schedule is recommended for chloromycetin in veterinary medicine.

From the Parke-Davis & Company Research Laboratories, Detroit, Mich.

*Parke, Davis and Company.

MATERIALS AND METHODS

Four dogs were used for each chloromycetin® dosage schedule in this blood level study. The administration of chloromycetin was begun about twelve hours after the animals were given their daily feed and all food was withheld until the experiment was completed. Chloromycetin was administered by mouth, each dose being placed in large gelatin capsules for ease of administration.

In the present study, 50, 100, 150, and 400 mg. of chloromycetin per kilogram of body weight was administered over a 24-hour period, divided into four equal doses at six-hour intervals. One group of dogs received 150 mg. per kilogram of body weight over a 24-hour period, divided into three doses of 50 mg. per kilogram each at eight-hour intervals. All blood was drawn from the jugular vein. Blood samples were drawn prior to dosage and at zero, two, four, six, eight, and ten hours after the last dose of chloromycetin. The resulting blood serum was assayed by the microbiological and colorimetrical methods.

The microbiological assay[4] is based on inhibition of growth of *Shigella sonnei* as measured turbidimetrically. One μg. per cubic centimeter is the threshold of test sensitivity. The colorimetrical assay[5] is based on reduction of the nitro group of chloromycetin by titanous chloride, following which the resulting amine is determined colorimetrically. This determination includes inactive metabolic products of chloromycetin which contain the nitro group, as well as unchanged chloromycetin. All results are expressed as micrograms of chloromycetin-equivalents per cubic centimeter of serum.

RESULTS AND DISCUSSION

The analytical results are presented in table 1 and graphically illustrated in fig. 1, each value representing the average serum level of 4 dogs by microbiological and colorimetrical assay. No untoward reactions were noticed in any of the experimental animals including those receiving 400 mg. per kilogram of body weight.

From table 1, it is evident that the maximum serum level is found at the two-hour

TABLE I—Average Chloromycetin® Blood Serum Levels As Determined by Microbiological and Colorimetrical Assays

Time of sampling (after last dose)	Dose of chloromycetin administered (Mg. per Kg. body weight per day)									
	50		100		150		150		400	
	12.5 mg. per Kg. at 6-hour intervals		25 mg. per Kg. at 6-hour intervals		37.5 mg. per Kg. at 6-hour intervals		50 mg per Kg. at 8-hour intervals		100 mg. per Kg. at 6-hour intervals	
	Micro-biological	Color-imetrical	Micro-biological	Color-imetrical	Micro-biological	Color-imetrical	Micro-biological	Color-imetrical	Micro-biological	Color-imetrical
Hours	Chloromycetin equivalents in blood serum (μg. per cc.)									
0*	2.1	7.2	4.5	23.2	6.7	38.0	5.0	17.6	...	97.7
2	3.9	17.2	16.6	43.2	22.1	61.0	16.3	37.6	...	79.0
4	2.8	13.5	8.2	32.0	13.0	50.5	16.7	40.0	...	100.2
6	0.4	8.5	3.3	20.0	6.0	33.2	8.7	29.6	...	108.0
8	0.0	4.2	< 2.5	11.2	2.4	16.5	5.0	19.3	...	92.6
10	0.0	2.2	0.0	7.2	0.5	10.5	1.8	10.0	...	52.2

*Sample drawn immediately before last dose.

sampling period, indicating fairly rapid absorption of chloromycetin.® The levels appear to be roughly proportional to the dose given. With doses greater than 100 mg. per kilogram per day, low concentrations of chloromycetin are found in the serum as long as ten hours following the administration of the last dose. In all instances, the colorimetrical values are considerably greater than the microbiological assay values, because of the presence of inactive nitro compounds derived from chloromycetin.[3] The major portion of the drug exists as an inactive conjugate with glucuronic acid which is excreted in the urine and bile along with small amounts of unchanged chloromycetin.[6] Antibiotic activity is also found in the cecum and large intestine of the rat after administration of the glucuronide derivative, because of the hydrolytic action of bacterial enzymes. A portion of the liberated chloromycetin is then reabsorbed.[7] It is not unlikely that this process occurs in the dog. This process would contribute to the maintenance of effective chloromycetin levels in the blood and tissues, providing a cyclic mechanism for return of a portion of the metabolized drug to the body in an active form.[7]

Most chloromycetin-susceptible micröorganisms are strongly inhibited by concentrations below 2.5 μg. per cubic centimeter.[1, 8] Since chloromycetin is partly bound to serum proteins,[1, 3] a figure of 5 to 10 μg. per cubic centimeter of serum would appear to constitute a clinically effective blood sesum level. This is supported by clinical experience.[9, 10, 11] From table 1, 150 mg. per kilogram over a 24-hour period, divided into three or four doses, appears to be adequate for the maintenance of a therapeutic level over most of the period between doses. The three-dose schedule (50 mg. per kg. per dose at 8-hour intervals) seems to be satisfactory from the standpoint of maintenance of blood levels and convenience in administration. It is, therefore, recommended as a therapeutic dosage schedule.

SUMMARY

Chloromycetin® blood serum levels in dogs have been determined by microbiological and colorimetrical assay methods, following the administration of chloromycetin by mouth at rates of 50, 100, 150, and 400 mg. per kilogram of body weight per day in divided doses at six-hour intervals and of 150 mg. per kilogram of body weight in divided doses at eight-hour intervals.

Levels were roughly proportional to dosage. Therapeutic blood levels of 5 to 10 μg. per cubic centimeter were maintained over most of the period between doses by the

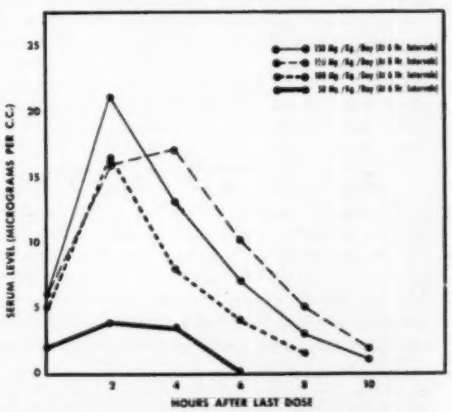

Fig. 1—Maintenance of chloromycetin® blood serum levels in dogs. (Each dose average of 4 dogs.)

administration of 150 mg. per kilogram of body weight over a 24-hour period, divided into three or four doses. No untoward reactions were noticed in any of the experimental animals including those receiving 400 mg. per kilogram of body weight.

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Blood Groups in Dogs — Their Significance to the Veterinarian

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STUDIES ON blood groups in dogs, initiated by Von Dungern and Hirschfeld[1] in 1910, have been pursued with renewed vigor in recent years, chiefly by investigators engaged in transfusing dogs for experimental purposes.[2-15] The object of this paper is to summarize the results of an intensive study of blood groups in dogs conducted over a period of four years at the University of Rochester Medical Center[11-16] and, in particular, to clarify the implications of these results for the veterinarian.

Specificity of Iso-Antibodies in the Dog.—Investigation of hemolytic reactions among dogs transfused at this Center without blood-grouping showed that the affected dogs had developed an antibody capable of agglutinating or hemolyzing the red cells of about 60 per cent of random dogs tested. This antibody, the first investigated, was called anti-A and the corresponding antigen in canine red cells was designated "canine A factor." Subsequent cross-immunizations have produced four other distinct iso-antibodies, designated anti-B, -C, -D, and -E in the order of their demonstration in this laboratory.

The frequency with which red cells from random mongrels react with five types of canine antiserums is given in table 1.

The five antiserums react independently with red cells of the dog. The frequency of combinations of antigens determined by these antiserums in 138 random dogs is given in table 2. Studies of inheritance suggest that each factor is gene-determined, but there is no evidence that any of the factors are linked or allelomorphic to any other factor. Although our studies have not yet extended beyond random mongrel dogs, we expect that interesting data will be obtained when the blood groups of purebred dogs can be investigated.

PROCEDURE

"Naturally Occurring" Iso-Antibodies in the Dog.—The problem of "naturally occurring" antibodies in dog serum has long perplexed both veterinarians and serologists. Claims have been made that such antibodies are not encountered and that transfusions can be performed at will among dogs. Various investigators,[6, 7, 10] on the other hand, have reported demonstration of naturally occurring iso-antibodies in as many as 50 per cent of dogs by use of special techniques. The discordant results thus far reported are presumably attributable to differences in technique and possibly to confusion with nonspecific cold agglutination and rouleaux formation which may, at times, be very troublesome to the serologist. The possibility that iso-antibodies might be formed in some dogs following artificial immunization against infectious diseases, such as distemper, has not yet been investigated.

In examining the serums of 145 random, nontransfused dogs, we were able to find naturally occurring iso-antibodies in the serums of 22 dogs. The serums tested were mixed at room temperature with equal volumes of 4 per cent suspensions of red cells in autogenous serum from a selected panel of dogs of known blood type. Each serum tested was also mixed with autogenous red cells in order to measure its capacity for cold agglutination and rouleaux formation.* The naturally occurring iso-antibodies in 14 of the 22 serums appeared to be of anti-D specificity; 3 were of anti-B specificity and, in 5 of the serums, the antibodies were of specificities that have not yet been identified. After storage of the serums at 4 C. for twenty-four hours, the naturally occurring iso-agglutinins frequently could no longer be demonstrated, even though the serums produced three plus to four plus reactions when tested within one to four hours after being drawn. The titer of the agglutinin in nontransfused dogs was rarely more than 1:8, despite the fact that strong reactions were frequently observed with undiluted serum.

Only those undiluted serums producing at least one plus agglutination that could not be dispersed with moderate agitation of the tube were considered positive in the routine screening tests for naturally occurring iso-agglutinins. Easily dispersed agglutination was often encountered, but such reactions could not

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*We have not found it advantageous to use serum albumin as a diluent or cell-suspending medium, as described by Hamilton.[10] Olsen's observation[6] that naturally occurring dog iso-antibodies were much more readily demonstrable in plasma than in serum could not be confirmed in our laboratory.

TABLE 1—Frequency of Reaction of Erythrocytes from Random Dogs with Five Types of Dog Antiserums

Serum	No. dogs tested	No. dogs positive	Positive (%)
Anti-A	460	290*	63
Anti-B	460	28	6
Anti-C	460	454	99
Anti-D	321	87	27
Anti-E	138	110	80

*This figure includes both A and A' dogs. A' cells are defined as those which, when tested against a battery of anti-A serums, are not directly agglutinated by all of the antiserums but are regularly sensitized for the antiglobulin reaction by nearly all anti-A serums.

be reproduced with any regularity and their specificity could not be adequately investigated. We have been unable to determine with certainty whether or not reactions of this sort are due to extremely weak, naturally occurring iso-agglutinins. Experience to date indicates that the naturally occurring canine iso-antibodies, as well as canine anti-B, -C, -D, and -E in transfused dogs, are of little or no importance with respect to transfusion incompatibility. For example, in a recent experiment, 150 cc. of D-positive blood were given to a D-negative recipient with serum containing natural anti-D in a titer of 1:8. A battery of tests employed after the transfusion revealed no readily demonstrable *in vivo* destruction of the transfused D-positive red cells, despite the fact that these cells apparently absorbed all of the anti-D in the recipient's circulating blood plasma.

Antigenicity of Blood Group Factors in Dogs.—Data on development of iso-antibodies in transfused dogs are summarized in table 3. In most instances, 5 to 20 cc. of whole citrated blood were injected intravenously once or twice weekly during the period of immunization. Anti-A developed in substantial titers within forty days in most of the A-negative dogs transfused with A-positive cells. Anti-A appeared in nine to fourteen days in 3 dogs after a single transfusion. Despite the fact that C-negative dogs are a rarity (1% of random mongrels), we have been impressed with the ease with which anti-C develops in such dogs after transfusion of C-positive cells. We have had opportunity to study the development of such an antibody in 4 dogs, 2 of which were highly immunized after only one or two injections of C-positive red cells. Experience with the production of other canine iso-antibodies has shown that, in general, they develop somewhat more slowly than anti-A and anti-C.

TABLE 2—Frequency of Combinations of Agglutinogens in 138 Random Dogs

Agglutinogens	No. of dogs	Agglutinogens	No. of dogs
ACE	52	AE	2
CE	23	BCE	2
ACDE	13	ABC	1
AC	10	ABE	1
C	10	ACD	1
ABCDE	8	(?F)	1
CDE	5	....	..
CD	5	....	..
ABCE	4	....	..
Total No. of dogs			138

DISCUSSION

The data in table 1 can be used to predict the frequency with which each of the antibodies may be formed as a result of random transfusions (table 4). Since all canine red cell factors are not equally antigenic nor are all dogs capable of producing antibodies to these foreign antigens with equal facility, antibodies may not be formed after every transfusion in which there is a blood group difference. The A factor is of the greatest practical importance. Thirty-seven per cent of random dogs lack the canine A factor and 63 per cent of random donors for such dogs have red cells containing the A factor. By multiplying these frequencies (0.37 by 0.63 = 0.23), one can calculate that approximately 25 per cent of random transfusions have the potentiality for production of anti-A in the recipients. If a dog is transfused a second time and the donor is again chosen at random, one may similarly calculate that about 15 per cent will be sensitized with the first transfusion and given an incompatible transfusion the second time.

Further experience will be necessary before reliable estimates can be made regard-

TABLE 3—Data on Development of Iso-Antibodies in Transfused Dogs

Agglutinogen present in transfused red cells and lacking in recipients' red cells	No. dogs transfused	No. dogs developing iso-antibody	No. dogs developing iso-antibody in given No. of days after first immunizing transfusion			
			Days			
			8-14	15-40	41-100	101-206
A	23	21*	8	10	1	2
B	5	4	..	2	1	1
C	5	5	1	4	..	..
D	13	6	2**	4	..	..
E	3	2	1	..	..	1

*The 2 dogs that failed to develop anti-A after multiple transfusions of A-positive cells were thought to be A-negative. In retrospect, however, it is considered possible that these dogs had A' cells and were therefore incorrectly typed by the methods in use at the time. Both of these dogs died before newer methods of typing red cells were in use.

**One of the D-negative dogs that ultimately developed anti-D in high titer had a small amount of anti-D in the serum prior to transfusion.

ing the proportion of dogs that can be expected to produce iso-antibodies against any given canine agglutinogen. Experience already summarized in table 3 indicates, however, that perhaps 40 per cent of A-negative dogs will develop anti-A within fourteen days after transfusion of A-positive cells and that 80 to 90 per cent may develop anti-A within forty days if one or two small transfusions are given weekly. On the basis of these figures and those given in table 3, one can reasonably conclude that nearly 10 per cent of *all* dogs transfused at random might develop anti-A within fourteen days and perhaps 20 per cent would develop anti-A within forty days.

A′ Red Cells.—All A-positive red cells are not directly agglutinated by all anti-A serums. Red cells containing the A factor show various degrees of reactivity; some "weak" A cells (so-called A′ cells) may, in fact, show no agglutination even when mixed with potent anti-A serum. Coating of the A′ cells with anti-A antibody can be detected, however, by means of the antiglobulin test.[13] The antiglobulin reagent is a serum produced in rabbits by injections of normal dog serum, and is used to demonstrate small amounts of antibody fixed to the surface of the red cells. The antibody-coated erythrocytes of dogs are agglutinated by the antiglobulin rabbit serum, as indicated in fig. 1.

Recognition of A′ cells has a twofold importance. The presence of A′ cells in a recipient may not be detected by ordinary typing, so that an apparently A-negative dog will fail to develop anti-A antibody upon stimulation with A-positive cells. Secondly, A′ cells may be given to an A-negative recipient under the false assumption that they are A-negative. The recipient may then develop anti-A antibody. A′ cells, too weak to be detected with potent anti-A typing serum, survive in the immunized recipient for longer periods than "strong" A-positive cells but for a shorter period than A-negative cells.[16]

Hemolytic Transfusion Reactions Due to Canine Anti-A.—The canine iso-antibody designated anti-A hemolyzes A-positive red cells rapidly both *in vitro* and *in vivo*. When A-positive cells are transfused into a previously immunized A-negative recipient, most of the donated corpuscles may be destroyed within less than an hour, with consequent hemoglobinemia, hemoglobinuria, fever, emesis, incontinence, tremors, hives, and transient prostration.[12] The outward manifestation may be severe or in some cases so slight that the occurrence of a hemolytic reaction in the dog's circulation would not be suspected from casual observation of the dog's behavior. If the recipient has not been transfused previously, the donated cells may be destroyed as the anti-A antibodies develop after seven to ten days or more. Destruction of the donated cells in the latter case does not usually cause the dog to appear ill, but the recipient is nevertheless deprived of full benefit from the transfusion. The life span of canine erythrocytes is approximately the same as that of human erythrocytes.[16] Normal compatible cells have been shown to survive in both species for more than one hundred days.

When canine plasma containing anti-A is transfused into A-positive recipients, the recipient's red cells may be destroyed in

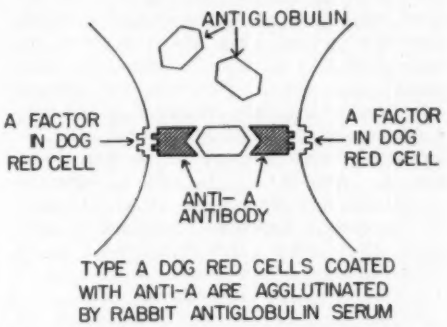
ANTIGLOBULIN
A FACTOR IN DOG RED CELL
A FACTOR IN DOG RED CELL
ANTI-A ANTIBODY
TYPE A DOG RED CELLS COATED WITH ANTI-A ARE AGGLUTINATED BY RABBIT ANTIGLOBULIN SERUM

Fig. 1—Diagram indicating manner in which red cells coated or sensitized with antibody, such as canine anti-A, may be agglutinated by antiglobulin serum. Modified from van Loghem, Kresner, Coombs, and Roberts (Lancet, 2, 1950:729).

TABLE 4—Estimates of Frequency of Potentially Immunizing Transfusions in Dogs Transfused Indiscriminately Without Respect to Blood Type

Agglutinogen	Random dogs having agglutinogen in red cells (%)	Random dogs lacking agglutinogen (%)	Random transfusions potentially immunizing (%)
A	63	37	23
B	6	94	6
C	99	1	1
D	27	73	20
E	80	20	16

large numbers over the following ten days.[16] Large volumes of plasma containing anti-B, -C, -D, and -E, on the other hand, may be given with relative impunity to dogs whose red cells contain the corresponding factors.[16] When B, C, D, and E cells are transfused into immunized recipients, the donated cells are apparently destroyed much more slowly than when there is an A incompatibility. Further studies on such transfusions are in progress and should provide more accurate estimates of the rate of destruction of donated dog red cells incompatible with respect to factors other than A. Anti-B, -C, -D, and -E strongly agglutinate dog red cells *in vitro* but cause no hemolysis. Repeated experiments have shown that these antibodies are of much less importance than anti-A in respect to *in vivo* destruction of erythrocytes.

Precautions Regarding Transfusion of Dogs.—Our experience to date indicates that only the canine A factor need be taken into account in planning blood transfusions for dogs. It is impractical to attempt to prevent development of iso-antibodies other than anti-A. If such antibodies develop, they can be detected by simple crossmatching of blood samples from the prospective donor and recipient (*see* Appendix). Even though it is unlikely that anti-B, -C, -D, and -E would cause serious hemolytic reactions, incompatibility with respect to these factors should forestall use of the proposed donor.

Incompatibility with respect to the A factor can be avoided by testing the red cells of the prospective recipient and donor dogs with potent anti-A serum and transfusing A-negative recipients with A-negative blood only. A-positive recipients could be given A-positive blood or A-negative blood which contains no anti-A antibody. Crossmatching of blood samples from donor and recipient (*see* Appendix) is an additional precaution worth taking, especially when either the recipient or donor dogs may have been transfused previously.

The simple precaution of using only A-negative dogs as donors should eliminate most of the hazard of hemolytic reactions. The additional precautions of typing the recipient and crossmatching donor and recipient will admittedly add little to the safety of transfusion in most instances.

If the prospective recipient dog has not been transfused previously, the risk of giving A-positive blood to an A-negative recipient by transfusing without respect to blood type may frequently be justified in emergencies. It should be appreciated, however, that there are three potential hazards in such a policy:

1) The donated red cells may be rapidly destroyed, after about the tenth post-transfusion day if anti-A antibodies develop in the recipient; additional transfusions may become necessary.

2) A single transfusion of A-positive red cells may immunize an A-negative recipient so that any subsequent transfusion of A-positive cells may cause an immediate hemolytic reaction.

3) An A-negative bitch transfused with A-positive blood and subsequently mated with an A-positive sire may transmit anti-A antibodies through her milk to pups, thus producing hemolytic anemia in the offspring, as will be explained in the following paragraphs.

Hemolytic Anemia in Newborn Dogs.—Newborn dogs, like foals and baby pigs, readily absorb maternal iso-antibodies from the mother's milk during the first day of life. We have thus far investigated 11 litters of pups born to A-negative bitches immunized with the blood of A-positive sires. Hemolytic anemia of varying severity was encountered in all A-positive pups that suckled an immunized bitch during the first day of life. Severely affected pups usually died within two or three days. A-negative pups were unaffected, but anti-A antibodies could be found in their serum after they had fed on milk containing antibody. None of the pups born to bitches immunized against the canine C (4 litters) and D (1 litter) factors developed hemolytic disease.

Hemolytic disease of newborn pups is caused by the ingestion of antibodies in the bitch's milk during the first twenty-fours of life. If the pups are kept from suckling during the first twenty-fours hours, they may then nurse freely, since they are apparently unable to absorb antibody from the mother's milk after the first day of life. During this period, they may be given cow's milk or allowed to suckle a foster bitch having no iso-antibodies in her milk. Since no iso-antibody has been demonstrable in the blood of pups at birth before they suckle, we may surmise that the dog placenta, like that of the horse[17] and pig,[18]

does not permit passage of antibodies from mother to offspring.

Abelson[19] has observed hemolytic disease in pups born to bitches that had not been transfused but had presumably been immunized by an antigenic factor in the red cells (or other tissues or body fluids) of the fetus. Natural immunization of this sort occurs in pregnant mares[17] and is common in Rh-negative women bearing Rh-positive fetuses. Veterinarians in Rochester have thus far submitted five litters of pups to our laboratory for examination because of a suspicion of naturally occurring hemolytic disease, but no evidence of this disorder could be found. Natural iso-immunization of the bitch during pregnancy is probably a rarity, but further observations are needed to clarify this matter. A-negative bitches may be readily immunized by transfusion, however, and hemolytic disease in pups might therefore be seen frequently if indiscriminate transfusion therapy should be used in treating canine casualties.

SUMMARY

1) Five different iso-antibodies have been demonstrated in serums from transfused dogs and designated anti-A, -B, -C, -D, and -E in the order of their demonstration. "Naturally occurring" antibodies have been found in about 15 per cent of random dogs that had never been transfused. More than half of these antibodies had an anti-D specificity, while others were of either anti-B or unknown specificity. Anti-A is a potent lysin *in vitro* and *in vivo*. The other antibodies agglutinate but do not hemolyze dog erythrocytes *in vitro*, and they appear to cause little or no damage to dog red cells *in vivo*.

2) The importance of considering the "canine A factor" in planning dog transfusions is stressed. Since 37 per cent of random dogs have red cells lacking the factor and 63 per cent of random donor dogs have red cells containing this factor, it can be calculated that approximately 23 per cent of random transfusions have the potentiality for production of anti-A in the recipients. The A factor has been found to be highly antigenic, sometimes causing appearance of anti-A within nine days after a single transfusion. The A factor occurs in many degrees of reactivity and the weaker antigen (called A′) may not be detected in routine typing of dog red cells.

Differences between donor and recipient dogs with respect to antigenic factors other than A can usually be ignored. Routine crossmatching is recommended, however, for detection of agglutinins other than anti-A, despite the fact that the donor and the recipient dogs are not typed for all the antigens known to be present in canine red cells. This practice is similar to that followed in planning human blood transfusions in which only the ABO groups and the Rh type are taken into account.

3) Hemolytic anemia has been observed regularly in A-positive pups born to A-negative bitches immunized by transfusions of A-positive blood. All anti-A iso-antibody is apparently acquired by the pups from the bitches' milk during the first day of life. Natural iso-immunization of the bitch during pregnancy (without transfusion) may occur and is a problem deserving further investigation.

Appendix

1) *Technique of Testing Red Cells of Dogs with Anti-A Canine Serum.*—On the basis of available information, it is currently recommended that red corpuscles of prospective donor and recipient dogs be tested only for the presence or absence of the canine A factor. The anti-A serum used in our laboratory is obtained from A-negative dogs that have been immunized by transfusions of A-positive dog red cells. The typing serum for use in the slide method is prepared by mixing one volume of serum inactivated by heating at 56 C. for thirty minutes with one-tenth volume of 0.85 per cent sodium chloride solution containing 1.0 per cent sodium azide. Thirty

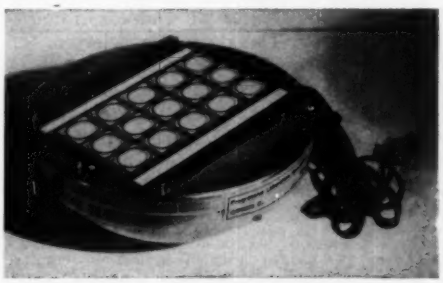

Fig. 2—Glass slide and viewing box found most satisfactory for typing canine red cells with anti-A serum.‡

‡Photograph supplied by Progressive Laboratory Specialties Company, Jamaica, N. Y., manufacturers.

milligrams of potassium oxalate are added to each 10-ml. portion of serum in order to prevent formation of fibrin clots when the serum is mixed with whole blood containing a minimum of oxalate.

Venous blood samples of 5 cc. each are placed in tubes containing 6 mg. of ammonium oxalate and 4 mg. of potassium oxalate.** One drop of the oxalated blood sample and one drop of anti-A serum are thoroughly mixed using a clean toothpick on a well-illuminated glass slide (fig. 2). Separate, clean Pasteur pipettes should be used for serum and the blood sample. The mixture is examined for the presence or absence of agglutination after ninety to 120 seconds. Readings made after two minutes are frequently misleading because nonspecific aggregation of the cells occurs after this period. Red cells to be tested can also be mixed with typing serum in small test tubes as described later for crossmatching, but the slide method is more convenient and has the further advantage of causing a higher proportion of A′ cells to be directly agglutinated by anti-A serum.†

The large portion of the specimen of oxalated blood not used in serological tests can often be employed advantageously in other determinations such as hematocrit, hemoglobin, reticulocyte count, and plasma bilirubin concentration.

2) *Technique of Crossmatching Blood from Prospective Donor and Recipient Dogs.*—The crossmatching of canine blood is done after both the donor and recipient dogs have been typed and compatible types have been selected. Matching tests are carried out in small test tubes rather than on slides, in order to enable the examiner to detect hemolysis and to demonstrate any iso-agglutinins that might cause a transfusion reaction. The dogs are selected for probable compatibility on the basis of tests for the A factor prior to the crossmatch. Either A-positive or A-negative blood may be given to an A-positive recipient, but one must be certain that the serum of an A-negative donor does not contain an anti-A antibody. Only A-negative blood may be given to an A-negative recipient. Even though donor and recipient dogs are typed for the A factor, a crossmatch should be done routinely as an additional precaution to detect any iso-antibodies that might lead to a transfusion reaction.

The samples of clotted blood used in crossmatching should be drawn within a few hours of the time of performing the test. The clots should be centrifuged shortly after coagulation has occurred and before the clots have become so firm that free red cells can no longer be easily dislodged. The cell-free serum is withdrawn with a Pasteur pipette and divided into two parts. The first part is used unaltered and the other is used to make a 4.0 per cent suspension of autogenous cells. The cells may be freed by gently fragmenting the clot with a pipette tip or applicator stick. They are removed with a clean Pasteur pipette and added to the second portion of the serum in a proportion of about one drop of concentrated cells to about twenty drops of the serum.

Equal parts (usually 0.1 ml. or 2 drops) of the recipient's serum and the donor's cell suspension are mixed in a small test tube (approximately 7 by 60 mm.). In a second tube, equal volumes of the donor's serum and the recipient's cell suspension are mixed. Both tubes are allowed to stand for fifteen minutes at room temperature and are then spun in a centrifuge for one minute at approximately 500 to 1,000 revolutions per minute (r.p.m.), after which they are examined for hemolysis, shaken gently, and examined for grossly visible agglutination. Various degrees of nonspecific hemolysis may be found in dog blood, but a potent lysin can be expected to cause complete or nearly complete hemolysis of the test cells. If no agglutination is seen grossly, a drop of the mixture is transferred with a Pasteur pipette to a glass slide and examined under the low power of a microscope for the presence of agglutination. Both the donor and recipient cell suspensions should be examined in a similar manner after centrifugation for one minute at 1,000 r.p.m. to ensure that there is no hemolysis, auto-agglutination, or excessive rouleaux formation in the cell suspensions before they are used in the matching tests. Either significant hemolysis or agglutination in the crossmatch indicates an incom-

**The mixture of potassium and ammonium oxalates can be obtained from the American Hospital Supply Corporation, Evanston, Ill.

†In one series of tests on samples of oxalated blood from 124 dogs selected at random, 76 were found to be A-positive by the combination of tube testing followed by antiglobulin tests on cells not directly agglutinated by anti-A serum. The slide test detected 72 of the 76 A-positive samples, while the tube test done in parallel detected only 57.

patibility and the necessity of choosing a new donor.

3) *Production of Canine Iso-Immune Serum.*—The production of canine blood-typing serum containing an antibody of a single specificity should not be attempted unless the donor and the recipient dogs are tested for all known factors in their red cells. It is probable that a number of unidentified antigenic factors exist in dog erythrocytes and that, even though tests are made for all factors presently known, antibodies of more than one specificity may be produced. The recipient may thus form an antibody against a factor other than A present in the donor's red cells. Serum containing antibodies of more than one specificity can be adequately studied only by testing the serum against a large panel of dog cells of known type.

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Pathology of Spontaneous Canine Histoplasmosis. A Study of Twenty-One Cases

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HISTOPLASMOSIS IS a disease of man and lower animals caused by the fungus *Histoplasma capsulatum*. Disseminated and localized forms of the disease occur in man, but only the disseminated form has been described in lower animals. Two types of disseminated histoplasmosis are recognized in man and in lower animals. One type progresses rapidly and is usually fatal, while the other is more quiescent and its mortality seems not nearly so high, although no accurate data are available on either.

LITERATURE CITED

Darling[6] first described histoplasmosis in in 1906 when he found it in Panamanian natives. DeMonbreun[6] proved the causative agent was a fungus in 1934. The pathology of histoplasmosis in the human being has had rather thorough study. There is almost consistent hepatosplenomegaly and moderate enlargement of individual and chains of lymph nodes in the disseminated form.[1] Pathological changes have also been reported in most of the other organs of the body. In the localized form, ulcerative lesions are present on the skin and on the mucous membranes.

DeMonbreun[7] reported the first case of spontaneous canine histoplasmosis in 1939. He concluded that the organism recovered from the dog was identical to that which caused the disease in the human being and suggested that the dog might serve as a reservoir of infection for man. He produced histoplasmosis in puppies by feeding a mixture of yeast and mycelial cultures in milk. Disseminated histoplasmosis has also been produced in puppies by inoculating a saline suspension of mycelial cultures into the nasal passages.[10] These experimental results, together with data collected from the present series of cases, suggest the mouth as an important portal of entry of *H. capsulatum* in the dog.

Approximately, 25 proved cases of spontaneous canine histoplasmosis have now been reported. Histopathological changes were described in various organs of 11 of these cases, but only a few organs were available for microscopic study in most cases.[2, 3, 4, 7, 8, 13, 14, 15] The distribution of microscopic lesions reported from these cases are tabulated: stomach, 1; small intestine, 4; colon, 3; liver, 4; pancreas, 1; spleen, 7; peripheral lymph nodes, 2; thoracic lymph nodes, 2; bone marrow, 1; heart, 1; lungs, 4; adrenals, 1; and kidneys, 1. Microscopic descriptions of lesions had a remarkable similarity regardless of the individual case or organ.

Lesions were generally characterized by an increase in macrophages, many of which were parasitized with *H. capsulatum*. Necrosis of the greatly enlarged lymph nodes was observed frequently, and an occasional lesion was calcified. The microscopic appearance of canine lesions was very similar to that described in human histoplasmosis.

OBSERVATIONS IN PRESENT SERIES

The clinical manifestations as well as the anatomical distribution and character of lesions of spontaneous canine histoplasmosis were studied. Twenty-one cases were included in this series. Tissue was available for microscopic study from only a limited number of organs of some cases; therefore, information on these is incomplete. Nineteen of the cases originated within a radius of 60 miles of Nashville, Tenn., and the other two originated in east central Texas. Seventeen dogs were Boxers, and each was associated in some way with one or the other of two Boxer breeding kennels previously described.[11] Two were Cocker Spaniels, 1 was a Pekingese, and the other a mongrel. The ages ranged from 6 months to 4 years.

CLINICAL OBSERVATIONS

The onset of the clinical syndrome was usually gradual and insidious. A chronic cough, an irregular appetite, intermittent diarrhea, and a gradual weight loss were almost constant symptoms. Spreading of the posterior ribs and distention of the anterior abdomen were nearly always pres-

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ent. Some degree of anemia and icterus was frequently evident. Most of the foregoing manifestations increased in severity as the disease progressed. The body temperature was constantly elevated in each case and varied from 39.0 to 40.0 C. The animals finally became emaciated and died from five to nine weeks after symptoms were first observed.

Several definitive diagnostic methods were used in trying to establish a positive clinical diagnosis. These included blood cultures, examination of stained bone marrow films, and the histoplasmin skin test. Blood and tissue from 12 cases were available for culture, and *H. capsulatum* was recovered from each case. Stained bone marrow films from 8 cases were studied, and *H. capsulatum* was recognized in 4 cases. Eleven dogs with advanced disseminated histoplasmosis were skin-tested with histoplasmin and all were negative. Histoplasmin was used in dilutions of 1 : 10 and 1 : 100 in each case.

Pathological Manifestations

Pathological studies included the gross and microscopic alterations. Tissue was fixed in 10 per cent formalin and routinely stained with hematoxylin and eosin. In addition, several sections were stained by the Hotchkiss-McManus method for fungi. Postmortem examinations were performed on 11 cases at Vanderbilt Medical School. Pathological data on other cases were obtained from competent observers who also made various internal organs available for cultural and microscopic study.

Peritoneal Cavity.—Gross Appearance.—The mesenteries and omenta of 9 of the 12 dogs studied were thickened and granular on the surface. Excessive quantities of turbid fluid were present in the peritoneal cavities of several cases.

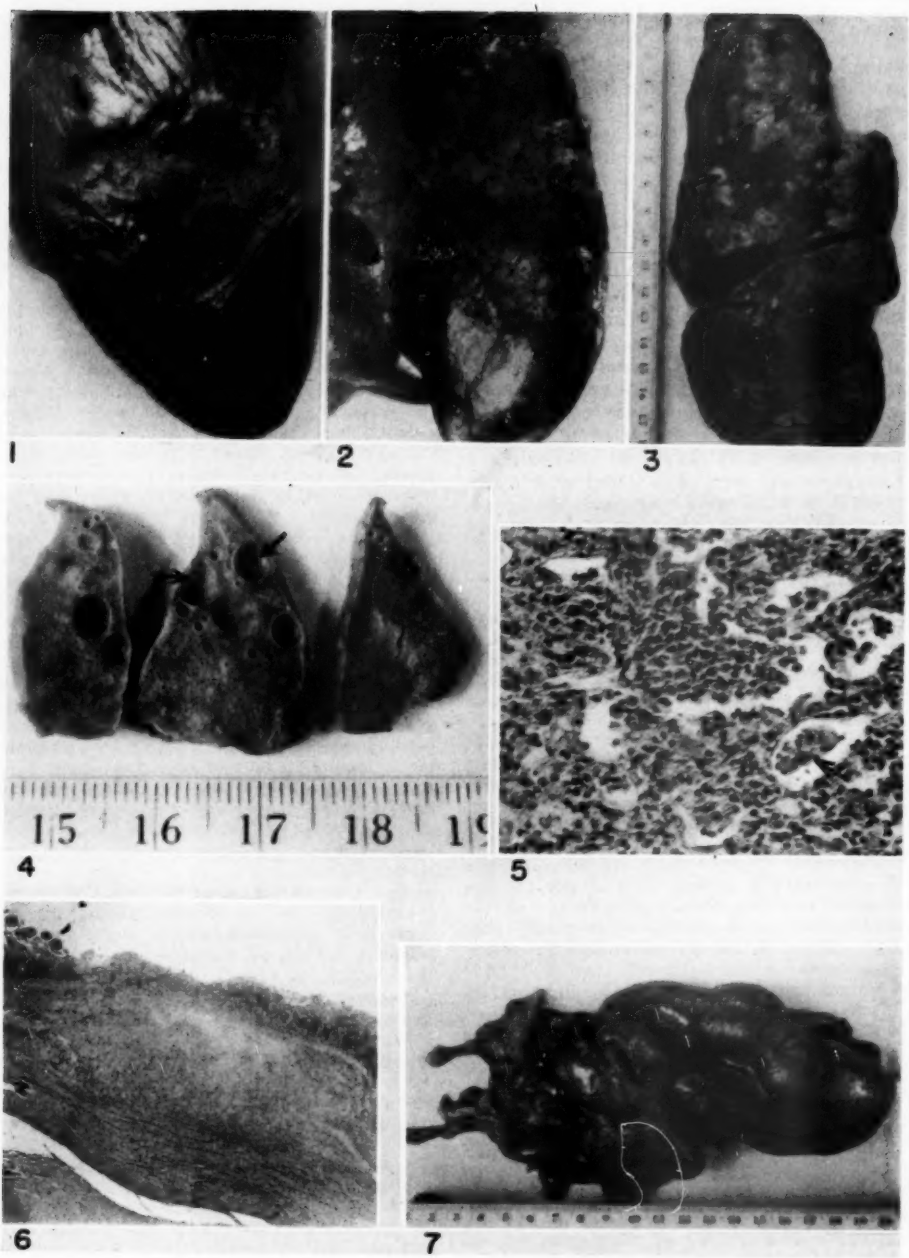
Microscopic Appearance.—An increase in connective tissue and macrophages produced the thickening of the mesenteries and the omenta. Necrotic foci and *H. capsulatum* were numerous in the tissue sections.

Pleural Cavity.—Gross and microscopic examinations revealed a thickening of the pleura of 1 dog; the pleural cavities of this case contained about 400 ml. of turbid fluid.

Cardiovascular System.—Gross Appearance.—Single circumscribed areas of myocardial degeneration were present in five of the thirteen hearts studied (fig. 1). All the lesions had the same general anatomical location. They were situated low in the lateral wall of the right ventricle and the adjoining septum, and immediately above the left coronary on the lateral surface, extending forward to the right anterior surface of the heart. They varied in size from 2 to 3 cm. wide by 3 to 4 cm. long, and they were somewhat irregular in shape. The myocardium in the defects was soft, flaccid, thin-walled, and the surface was shrunken below the level of the surrounding tissue. There was a white crusty layer of material, resembling calcium, in the endocardium of both auricles of three hearts. Similar lesions were present in the ascending aorta of 1 case. In each instance, the endothelium remained smooth and glistening, and no surface defects or thrombi were present.

Microscopic Appearance.—Tissue sections from the hearts of 11 dogs were studied and lesions were present in 10 cases. In the areas of myocardial degeneration, the muscle fibers were shrunken, frequently fragmented, and the striations were often dim or completely absent. The expanded intercellular spaces contained numerous round cells and some fibroblasts. Macrophages containing *H. capsulatum* were observed in the areas of myocardial degeneration in 2 cases, but these appeared to be equally numerous elsewhere in the myocardium. Many small hemorrhages usually were present in and around these large lesions. Necrotic foci were present elsewhere in the myocardium in many cases, and these were infiltrated with many round cells and occasionally with neutrophils. Foci of macrophages were also present in most hearts where necrosis was not visible. A layer of calcium was deposited between the endothelium and the myocardium of the auricles of 3 cases, and similar lesions were present in the aorta of 1 of these. The endothelium was intact and no thrombi were present over the lesions. A moderate number of macrophages, neutrophils, and fibroblasts were invading the myocardial border of the calcium, but necrosis was not visible. Plaques were present beneath the endothelium of the left coronary artery of one heart, but the lumen of the vessel was not occluded. Large macrophages were present in the plaques.

Respiratory System. — Gross Appearance. — Lesions were not observed in any of the upper respiratory passages, trachea, or bronchi in any of the 15 cases in which these structures were studied. The lungs of each animal had obvious pathological alterations. They were only partially collapsed and had a firm resistance. Evidence of focal consolidation was present in every case. The degree of consolidation varied from slightly firm lesions a few millimeters in diameter to solid nodules up to 3 cm. in diameter. The early focal lesions were dark red and had the appearance of fairly well-defined hemorrhages (fig. 2). These lesions measured up to 15 mm. in diameter and were fairly well distributed throughout the parenchyma. The large nodules were yellowish white and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had the appearance of metastatic neoplasms (fig. 3). These large solid nodules were present in the lungs of 3 mature dogs. Cavities were present in the lungs of 3 dogs, varying from 2 to 15 mm. in diameter (fig. 4). The cavities contained no necrotic material and there was no increase in connective tissue around them.

Microscopic Appearance.—The most basic pathological change in the lungs of each case was an infiltration of large numbers of macrophages. These cells formed masses in the interstitial tissue and plugs in many of the alveolar spaces (fig. 5). Many macrophages contained *H. capsulatum*. The organisms found in sections of lungs seemed to have a thicker capsule than those in other organs, and the stained portion of the yeast cell appeared smaller. Heavy concentrations of macrophages made up the firm areas and the solid nodules observed grossly. Macrophages containing *H. capsulatum* were seen free in the bronchial tree of almost every case. Neutrophils were present throughout the lungs in moderate numbers. There was no evidence of local inflammation present in the tracheae, bronchi, or bronchioles of any case. The parasitized macrophages present in the bronchial tree probably originated in the alveolar spaces. Large numbers of parasitized macrophages had infiltrated the borders of the pulmonary cavities, but there was no attempt to encapsulate the lesions.

Digestive System.—Gross Appearance.—There were shallow ulcers along the edges of the tongue near the tips in 2 cases in which this organ was examined. The stomachs of 12 dogs were examined and no lesions were observed. The wall of the small intestine was unevenly thickened in each of the 12 cases in which the gut was available for study. Two cases had only an apparent thickening of the mucosae, 4 had an uneven thickening of the entire walls, and 6 had well-defined, yellowish white plaques formed in the intestinal walls. The mucosa over the plaques was visibly ulcerated in 3 cases. The Peyer's patches were unusually prominent in each case.

Information is available on the gross appearance of the livers of 17 dogs. The organ was considered enlarged in each case. The degree of enlargement can be described only as moderate to extreme. Changes in all the livers were strikingly similar. The livers were mottled with light yellow, irregular-shaped splotches that varied from about 3 mm. to about 5 cm. in diameter. Considerable portions of some livers had an appearance compatible with multilobular cirrhosis.

No gross lesions were observed in the gall bladder or pancreas of any case.

Microscopic Appearance.—A moderate number of macrophages had infiltrated the tissue at the base of the glossal ulcers, and *H. capsulatum* was present in a few of them. Tissue sections from the stomachs of 4 dogs were studied, and all were free of lesions. Increased numbers of round cells were present in all strata of the wall of the small intestine of the 12 cases in which this structure was studied. Macrophages were numerous and many of these were parasitized with *H. capsulatum*. Lymphocytes and plasma cells were present in lesser numbers. The degree of invasion varied from a few cells to large masses which formed the plaques seen grossly. Necrosis of these plaques and ulceration of the mucosa were observed in 4 cases. The heaviest concentrations of invading cells were in the submucosa and usually involved the Peyer's patches (fig. 6). Macrophages parasitized with *H. capsulatum* were seen free on the mucosal surface in 6 cases. Microscopic lesions were observed in three of the five colons studied, and were similar to those present in the small intestine.

Large numbers of macrophages and an increase in connective tissue were present in each of the twelve livers studied. Lymphocytes were present in moderate numbers. These proliferative changes were most pronounced in the vicinity of the triads and along the interlobular stroma, but also extended into the lobules in severe cases. In many cases, there was an increase in the number of bile ducts. The normal liver architecture was seriously altered.

Legends for Illustrations on Opposite Page

Fig. 1—A triangular-shaped, infarct-like lesion in the myocardium of a dog. The wall is thin, flaccid, and shrunken below the surface of the surrounding tissue.

Fig. 2—Early pulmonary lesions of canine histoplasmosis. The lesions resemble hemorrhages.

Fig. 3—Advanced pulmonary lesions of canine histoplasmosis. The large nodules resemble metastatic neoplasms. Figure 5 is a microscopic view of one of these lesions.

Fig. 4—Cavities in a diaphragmatic lobe of the lungs of a fatal case of canine histoplasmosis. No exudate or connective tissue capsule was present.

Fig. 5—Infiltration of macrophages in an advanced pulmonary lesion. Yeast cells of Histoplasma capsulatum were present in most of the invading cells. This is also characteristic of early pulmonary lesions but to a smaller degree. Approximately x 300.

Fig. 6—A mass of fungus-laden macrophages infiltrated into the wall of the small intestine. Local destruction of the mucous membrane resulted in ulceration. Approximately x 35.

Fig. 7—A chain of greatly enlarged mesenteric lymph nodes which have coalesced to form a single neoplastic-looking mass.

and much of the parenchyma was destroyed in each case. Degenerative changes were present in most of the hepatic cells that remained. Sections of gall bladder were studied from 2 cases, and foci of macrophages containing *H.*

TABLE I—Study of Organs and Lesions Available for Microscopic Study from Cases of Spontaneous Canine Histoplasmosis

Organ	No. organs studied	No. organs with lesions
Tongue	2	2
Stomach	4	0
Small intestine	12	12
Colon	5	3
Liver	12	12
Gall bladder	2	2
Pancreas	7	4
Spleen	12	12
Peripheral lymph nodes	12	12
Thoracic lymph nodes	15	15
Abdominal lymph nodes	14	14
Tonsils	5	4
Bone marrow	5	5
Adrenals	11	10
Kidneys	10	6
Heart	11	10
Lungs	15	15
Testes	2	2

capsulatum were present in the wall of this organ in each case.

Microscopic lesions were present in the pancreas of 4 of the 7 cases in which this organ was studied. Lesions consisted of groups of macrophages, yeast cells of *H. capsulatum*, and an increase in interstitial connective tissue.

Hemopoietic System.—Gross Appearance.—The peripheral lymph nodes were considered enlarged in 10 of the 12 cases studied. The nodes about the head were enlarged more consistently and to a greater degree than any other external group. The thoracic nodes were enlarged in each of the 14 cases in which they were studied. In 2 cases, enlarged hilar nodes were causing serious obstruction of the large bronchi, and they were compressing the esophagus in another. The mesenteric nodes were enlarged in each of the 14 cases in which they were studied. These nodes frequently adhered to each other to form large neoplastic-looking masses (fig. 7). In 1 case, a mass of mesenteric nodes obstructed the lumen of the small intestine by compression from without. Incisions through the enlarged mesenteric and thoracic nodes often revealed necrosis and a heavy connective tissue stroma, but the necrotic lesions were poorly encapsulated.

The spleens were considered enlarged in 8 cases and within the normal size range in 4. Necrotic foci were not observed in the splenic parenchyma of any case.

The tonsils were considered enlarged in 2 of the 12 cases in which these structures were examined. The tonsils of 1 of the 2 dogs had several small ulcers on the surface.

Microscopic Appearance.—The primary causes of enlargement of all nodes studied were large numbers of macrophages and moderate proliferation of connective tissue. Large numbers of macrophages contained *H. capsulatum*. The architecture was so severely altered in the internal nodes that the tissue could seldom be identified. Calcification of necrotic lesions was not observed in this series.

The primary change observed in each spleen was a proliferation of reticulo-endothelial cells, and the major difference observed in the various spleens was the degree to which these cells had increased in number. In most spleens, the follicular structures had been seriously altered or completely destroyed. Many of the large mononuclear cells contained *H. capsulatum*.

Yeast cells of *H. capsulatum* were present in massed macrophages in the tonsils of 4 of the 5 dogs in which these structures were studied microscopically.

Bone marrow sections from 5 dogs were studied in addition to the stained bone marrow films examined before death. There was an increase in reticulo-endothelial cells in every case, and these were so numerous, in some cases, that the fat was completely replaced. Many yeast cells of *H. capsulatum* were present in each section.

Endocrine System.—Gross Appearance.—The adrenal glands were considered enlarged in 6 of the 11 cases in which they were examined. The configuration was obviously disturbed in only 1 case; gross focal lesions were visible in none. No lesions were observed in any of the other endocrine organs.

Microscopic Appearance.—Sections of adrenal glands from 11 cases were studied, and lesions were observed in 10 of them. Foci of macrophages had invaded the cortex and the medulla in almost every instance, and many of the invading cells contained *H. capsulatum*. Necrotic foci were observed in sections from 2 cases.

Urinary System.—Gross Appearance.—Gross changes in the kidneys were vague and indefinite. In most cases, the kidneys were light yellow and the intensity of the color varied considerably in each kidney. No significant changes were observed in the other structures of the urinary system.

Microscopic Appearance.—Kidneys of 10 dogs were studied microscopically and lesions were observed in 6 cases. Numerous masses of macrophages had invaded the interstitial tissue, and there was a considerable increase in the cellular content of the glomerular tufts. Macrophages containing *H. capsulatum* were seen in the glomerular tufts and in the interstital tissue of 5 cases and only in the interstitial tissue of the sixth case.

Genital System.—Gross Appearance.—No significant alterations in the genital organs were noted.

Microscopic Appearance.—A microscopic study

of the testes was made in only 2 cases, and foci of macrophages were present in the proliferated interstitial tissue of each of them. Yeast cells of *H. capsulatum* were observed in many of the macrophages. A few parasitized macrophages were seen in the lumens of the seminiferous tubules in each case. It was not determined if the organisms were in the tubules before death or if they were misplaced during the preparation of the slides.

Nervous System.—No gross or microscopic lesions were observed in any structures of the nervous system.

SUMMARY

Data from 21 cases of spontaneous canine histoplasmosis are summarized in this study. Tissue was available for microscopic study from only a limited number of organs of some cases, and the results of gross observations were often incomplete. For the sake of uniformity of results, organs and lesions were included in table 1 only when they were available for microscopic study.

Single circumscribed areas of degeneration were present in approximately the same anatomical location of the myocardidium of five of the thirteen hearts studied. A layer of calcium was deposited beneath the endocardium of the auricles of three hearts, and in one ascending aorta without evidence of previous necrosis. The mediums of many small arteries of 1 case with subendocardial lesions were also calcified.

Gross and microscopic lesions were present in the lungs, thoracic and abdominal lymph nodes, and small intestines of each case studied. The gastrointestinal tract is probably the most common portal of entry for *Histoplasma capsulatum* in dogs. In this event, the invading organisms reach the bronchi by way of the blood stream and lungs from the intestines. The gastrointestinal tract is then subjected to constant reseeding from swallowed bronchial exudates. The consistent intestinal lesions account for the large numbers of *H. capsulatum* recovered from the stools of several cases in recent studies.[12] The spleen was considered enlarged in 8 cases and normal in size in 4; however, microscopic lesions were present in all twelve spleens. No lesions were found effectively encapsulated in any organ.

The disseminated form of histoplasmosis seems to affect organs of one or more systems of the dog without any suggestion of specific clinical or anatomical syndromes.

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Arsenamide in the Treatment of Canine Filariasis

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FOLLOWING THE introduction of the phenylarsenoxide, arsenamide (p-[bis-(carboxymethylmercapto)-arsino] -benzamide), by Otto and Maren,[9] as an effective agent for the treatment of heartworm (*Dirofilaria immitis*) infection in the dog, this study was undertaken with the desire to simplify therapy by *per os* administration. In practical therapeutics, only the parenteral routes have been employed for the administration of the phenyl arsenoxides; however, experimental laboratory infections of *Trypanosoma equiperdum* in the rat[13] and syphilis in the rabbit[13, 1] have been successfully treated by the oral administration of mapharsen.

MATERIALS AND METHODS

The study, as conducted, was essentially a comparison between the oral and the intravenous routes for the administration of arsenamide in the treatment of heartworm infection of dogs. The comparison was made on the basis of therapeutic effectiveness and arsenic determinations. The quantitative determination of the urinary and fecal excretion and tissue concentration of arsenic was undertaken to evaluate the extent of intestinal absorption, which was regarded as a possible limiting factor in oral administration. Since absorption was not a factor when the intravenous route was employed, the foregoing arsenic determinations associated with intravenous administration were used as the base line values for comparing similar determinations following oral administration.

In carrying out the study, 11 infected dogs were treated intravenously and 9 were treated orally. The basic course of treatment with both routes of administration consisted of 15 daily doses of the drug, and the dogs were routinely destroyed forty-eight hours after the final dose for observation on the therapeutic activity against the adult heartworms. Included in the intravenous group were a limited number of trials in which the number of daily injections was reduced to ten, and the dogs were destroyed forty-eight hours after the final dose, which would correspond to the twelfth day of treatment in the 15-day trials. For the comparison of the two routes of administration on the basis of the arsenic determinations, dogs receiving the 15 daily doses were used. The total urinary and fecal output, during the complete course of treatment and the 48-hour period until the disposal of the dog, was collected for arsenic analysis. The tissues were sampled at necropsy for subsequent analysis for arsenic. A limited number of normal dogs, *i.e.*, not infected with heartworms, were also utilized for the arsenic determinations associated with oral administration.

The laboratory rat was used to obtain a preliminary evaluation of the intestinal absorption of arsenamide by comparing the urinary excretion of arsenic following a single oral dose with that following a single intravenous dose. Carbarsone oxide and mapharsen were included with arsenamide in this phase of the study. Three experiments were completed in which single doses of 1 per cent solutions of the compounds were given by stomach tube, and one experiment was completed in which single doses of 0.5 per cent solutions of the drugs were injected intravenously. The rats were confined to a metabolism apparatus for the collection of the urine for a period following drug administration. The apparatus consisted of a wire mesh cage containing the rats suspended over a large glass funnel which was fitted near the neck with a watch glass. This served to retain the feces but allowed the urine to drain into a collecting tube below.

For the intravenous therapy of the heartworm-infected dogs, a 1 per cent solution of the disodium salt of arsenamide was used. The solution was prepared according to the method outlined by Maren,[5] and a solution was made up fresh just prior to the start of a course of treatment. The injections were made into the radial or saphenous veins.

In the oral administration trials on the dogs,

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the free-acid form of arsenamide was given. Three methods were employed to administer the drug powder: (1) mixing with the daily meal, (2) in gelatin capsules, and (3) in enteric-coated capsules. To administer the drug with the daily meal, the powder was mixed with a portion of horse meat. After this was consumed, additional meat and dog meal was provided. The swallowing of the gelatin capsules was forced and the daily meal was provided immediately thereafter. When the enteric-coated capsules were given, a delay of two hours ensued before the daily meal was made available.

The dogs on which the arsenic determinations were completed were confined to a metabolism cage to insure the collection of the urine specimens. The cage was provided with a spouted, metal tray bottom and a superimposed steel mat which prevented contact with the tray. However, for the most part, only female dogs were utilized and an attempt was made to collect all of the urine by catheter. Ordinarily, daily catheterizing at 9 a.m. and 5 p.m. prevented the voiding of urine in the cage. When the feces was saved for arsenic determination, the specimen was collected from the cage or from the floor of the room in which the cage was located. The latter was the usual procedure, since the feces were characteristically voided during the exercise period that was permitted immediately following the routine morning catheterization. Both the cage and the floor of the room were thoroughly washed daily. The urine and fecal specimens were stored in the refrigerator at 5 C. until sampled for the arsenic analysis. In collecting the tissues for arsenic analysis, the samples were weighed directly into test tubes at the time of the necropsy and an overlay of 2 per cent formalin was added as a preservative.

Magnuson and Watson[4] was followed in making the arsenic analyses. The method's sensitive range, being 1 to 100 μg. of arsenic, made it necessary to adjust the size of the various samples to stay within the useful range in applying the method throughout this study. Standard reference curves, based on the recovery of known amounts of arsenic, were prepared for each type and size of specimen that was analyzed. To keep a constant check on the technique, control samples containing known amounts of added arsenic were routinely included with the determinations on the unknown samples. Either 1 or 2 cc. of rat urine constituted a sample. Routinely, 5 cc. of the dog urine was used. Triplicate determinations were made on each urine specimen, and the average was taken to compute the total arsenic content of a specimen. The fecal samples ranged in size from 0.5 to 2.0 Gm. Before sampling a fecal specimen, water was added and thoroughly mixed until a smooth, pasty consistency was obtained. Double distillation was routine for the fecal analyses to avoid phosphorous interference, and triplicate determinations were averaged to calculate the total arsenic content of a specimen. To determine the arsenic content of the dog tissues, a single analysis was completed on each tissue. The samples ranged in size from 5.0 to 10.0 Gm., except for the thyroid and adult filariae which were necessarily smaller. The color readings were made with a Coleman universal spectrophotometer, model 11, set at 790 mμ, plus filter (No. PC-5), and cells with a light path of 40 mm. were used.

The effectiveness of a course of therapy on an infected dog was based on the viability of the adult heartworms that were recovered at necropsy. Activity was taken as the index of viability. Immediately after the dog was destroyed, the heart and lungs were removed intact, and the right heart and pulmonary artery, including the finer branches, were opened in the search for the worms. As the adult worms were found, they were removed and placed in normal saline. Observations on activity were made on removal; however, final judgment was withheld until the saline containing the worms was kept under a desk lamp for at least one hour. Most of the worms that were recorded as active were quite active on removal, and this activity was maintained during the ensuing observation period. The worms that were classified as alive but noted as sluggish showed no activity on removal, but very weak contractions, characteristically of the anterior extremity, were detected after the worms were placed under the lamp. The worms that were judged to be dead either failed to exhibit any activity on removal (even to tactile stimuli after they had been kept under the lamp) or were recovered as fragments.

Observations were made on the adult female worms that were recovered from intravenously treated dogs to ascertain the status of the microfilariae *in utero*. These microscopic examinations were made both on the intact worm and after the anterior portion had been cut into small segments and the microfilariae expressed by gentle pressure under a cover slip. Living, dead but intact, and fragmenting female worms were included in these observations.

Microfilarial counts were made on the peripheral blood of the infected dogs before, midway, and at the termination of a course of treatment. The technique for estimating the number of microfilariae was essentially that described by Franks *et al.*[2] One cubic centimeter of blood was diluted to 4 cc. with 2 per cent formalin, mixed thoroughly, a 0.05- or 0.1-cc. sample removed with a 0.25-cc. tuberculin syringe and spread out on a slide under a cover slip. The counts of three such preparations were averaged to compute the number of microfilariae per cubic centimeter of the undiluted blood.

RESULTS

THERAPEUTIC ACTIVITY—INTRAVENOUS ADMINISTRATION

Death of the adult heartworms was regarded as the primary objective of therapy, while little significance was attached to following closely the effect on the circulating microfilariae. Previous work[9] indicated that 15 daily injections of arsenamide at dose rates as low as .23 mg. of As per kilogram of body weight (1.15 mg. of arsenamide per kilogram) were regularly lethal for the adult worms, whereas doses as high as .90 mg. of As per kilogram failed to eliminate the microfilariae from the peripheral blood during the course of treatment.

Adult Filariae.—The observations on the therapeutic effectiveness with the intravenous use of arsenamide in this study are summarized in table 1. Daily doses of .45 mg. of As per kilogram for fifteen days, which has been suggested[8] as the schedule for the treatment of heartworm infection, were administered to 3 dogs. Only dead worms were recovered from 2 of these dogs (No. 20 and 33), and only 2 living worms out of a total of over 26 worms were observed in the remaining dog (No. 29). Lowering the dose to .23 mg. of As per kilogram (dog 21) for 15 daily injections failed to kill the heartworms. The intermediate dose of .30 mg. of As per kilogram for fifteen days resulted in the recovery of only living worms from 1 dog (No. 30) and two dead and 10 living worms from another dog (No. 31).

Reducing the number of daily injections to ten in the remaining trials was prompted by the observation of Otto and Maren[9] that an eleven-day course of treatment with arsenamide resulted in the recovery of only dead heartworms at autopsy. Further suggestive of the effectiveness of shortened courses was evident in Jackson's[3] noting the development of a cough as characteristic during the second week of arsenamide therapy, which could suggest the inception of the lung damage following the death or injury of the adult worms.

Three dogs received a course of treatment consisting of ten daily injections at doses of .45 mg. of As per kilogram. Essentially, only dead worms were recovered from 1 dog (No. 27) of this group, in that a sluggish female was the only living worm present among the fragments of dead worms. Another dog (No. 28) presented 6 active worms and 1 sluggishly active living worm at necropsy, along with several fragments of dead worms. No worms were found in the remaining dog (No. 25) of this group.

Increasing the daily dose to .60 mg. of As per kilogram for the ten-day period in 1 dog (No. 32) was associated with the recovery of 6 sluggish worms and over 26 dead worms.

One additional dog (No. 19), not included in the tables, received a course of intravenous therapy with an arsenamide solution that had been prepared eighteen months previously and stored at 5 C. in a rubber-stoppered amber bottle. This was the only exception in the series to the use of freshly prepared solutions. Fifteen daily doses of .45 mg. of As per kilogram were given and only 6 living heartworms were recovered at the postmortem examination.

Microfilariae.—It will be noted that the microfilarial counts on the foregoing series of intravenously treated dogs are also included in table 1. In none of the dogs were the microfilariae eliminated from the peripheral blood during treatment, and the routine destroying of the dogs at forty-eight hours after the last dose precluded prolonging these observations. For the

TABLE I—Therapeutic Activity of Intravenous Courses of Arsenamide Against Dirofilaria Immitis

Dog (No.)	Dose rate (mg. As/kg.)	No. of daily doses	Microfilariae/cc. blood		Adult worms recovered			
					Living		Dead	
			Pretreatment	Post-treatment	Male	Female	Male	Female
20	.45	15	2,200	2,500	0	0	2 (F)	4
33	.45	15	44,600	49,800	0	0	3	9
29	.45	15	5,200	8,000	2	0	13 (F)	11
21	.23	15	100	6,000	25	14	0	0
30	.30	15	80	240	13 ; 1*	2	0	0
31	.30	15	8,500	61,000	2	8	2	0
25	.45	10	60	35	0	0	0	0
27	.45	10	3,800	3,400	0	1*	7 (F)	0
28	.45	10	3,400	3,500	2	4 ; 1*	0 (F)	0
32	.60	10	8,600	28,400	0	6*	20 (F)	6

*Sluggish. F = fragments of worms in addition to whole worms enumerated.

most part, the courses of treatment producing complete or appreciable therapeutic activity against the adult heartworms were associated with essentially no change in the post-treatment microfilarial count as compared with the pretreatment count, whereas those courses showing little or no lethal activity against the adult worms were, in general, related to increases in the microfilarial count during treatment. The latter association was especially marked in 2 dogs (No. 21 and 31).

Microfilariae in Utero.—The characteristic selective activity of arsenamide of killing the adult worms without effecting the elimination of the circulating microfilariae during treatment motivated the observations on female worms recovered from treated dogs to ascertain the status of the microfilariae *in utero.* Microscopic examinations were made on adult females, including sluggishly alive, dead, and fragmenting worms, that were recovered from 3 (No. 29, 32, and 33) of the foregoing series of intravenously treated dogs. Actively motile microfilariae were noted in all. Of particular interest were the living embryos persisting in the anterior end fragments of dead and disintegrating female worms.

Reactions Following Intravenous Administration.—The schedules of intravenous therapy with arsenamide were generally well tolerated. Symptoms that might be attributed to drug toxicity were limited to the loss of appetite in 4 dogs and single instances of loose bowel movements in 2 dogs. Two dogs (No. 27 and 31) exhibited poor appetites throughout the series of injections, 1 (No. 29) had a transient loss of appetite midway in the course, and 1 (No. 33) experienced a loss of appetite during the terminal part of treatment. Two dogs (No. 19 and 30) were in advanced pregnancy when therapy was started, and both pregnancies were terminated during the course of treatment with the birth of litters which were living and normal in all discernible respects. Death of the adult heartworms and the resulting pathology which was observed in the lungs was not manifested by any definite symptom. A suggestive relationship was noted in several dogs (No. 27, 29, 32, and 33), in which a seemingly unusual listless attitude developed during the second week of therapy. The tolerance to the lung damage seemed remarkable, especially in the cases where a relatively large number of worms had been killed and the resulting embolism, inflammatory reaction, and infarction were extensive. Characteristically, the damage was located in the diaphragmatic lobes; however, in the heavier infections, the other lobes were also involved.

Perivascular leakage occurred at various times and, without exception, the resulting reaction was localized in the affected limb. The reactions consisted of edematous swelling and tenderness, which gradually subsided without abscess formation until, after several days, only sclerotization remained at the immediate site of the leakage.

Arsenic Determinations on the Rat

The laboratory rat was used to obtain a preliminary estimate of the extent of the intestinal absorption of arsenamide. For comparative purposes, two additional phenyl arsenoxides, mapharsen and carbarsone oxide, were included in this phase of the study. A total of three experiments were completed in which single doses of the compounds were administered intragastrically, and one experiment was completed in which single doses of the compounds were given intravenously. The study was limited to the determination of the urinary excretion of arsenic for the 48- or 72-hour period following drug administration. Extending the study to include fecal and tissue determinations of arsenic was not carried out, because of the early finding that the normal rats used as controls contained appreciable quantities of arsenic in their tissues. The finding of the so-called "normal arsenic" resulting from a low grade dietary intake has also been reported for the rat by Wright *et al.*[15] and Rodman and Wright.[12]

Excretion Following Single Oral Doses.—In the three experiments with the three compounds administered intragastrically, the excretion of arsenic in the urine followed the same general pattern (table 2). The total arsenic content of the doses of the different compounds that was accounted for in the urine was of the same general order of magnitude, ranging from nearly 4 to 10 per cent in the 72-hour studies. Although not indicated in the data, the absorption of the compounds began soon after administration, since measurable quantities of arsenic were already present in the

urine specimens collected two hours subsequent to dosing. Appreciable arsenic recoveries were maintained through the third day; however, there was a tendency toward the decline by this time, which indicates that the initial acute phase of urinary arsenic excretion was nearly accounted for when the collections were terminated. The recovery of 5 to 8 per cent of the oral doses of mapharsen in the form of arsenic in the urine compares closely with the 9 to 10 per cent previously recorded[1] following oral doses of mapharsen in the rabbit.

Excretion Following Single Intravenous Doses.—A similar urinary excretion study following the intravenous injection of single doses of the foregoing compounds is summarized in experiment 4 of table 2. With each compound, the greater part of the arsenic that was recovered in the urine appeared in the eight-hour specimen immediately following the injection. Thereafter, the recoveries were markedly reduced until comparatively small concentrations of arsenic were observed in the 24- to 48-hour specimens. The total recovery of 35 per cent of the arsenic administered in the form of arsenamide, being nearly twice that recovered with mapharsen and carbarsone oxide, may be an extreme of the normal variation appearing in this single trial rather than an index of a true relationship. The 24-hour urinary excretion of 18 per cent of the intravenous dose of arsenic administered as mapharsen agrees favorably with the mean value of 13.32 per cent reported by Voegtlin and Thompson[14] in a similar study on the intravenous administration of mapharsen to the rat.

The total excretion of arsenic in the urine, expressed as milligrams of arsenic per kilogram of body weight, was of the same general order of magnitude for both routes of administration of a compound. Since the oral dose rate was five times the intravenous rate, intestinal absorption of the compounds was on the order of 20 per cent.

Arsenic determinations on the urine specimens from the control groups, included in each of the experiments, are not recorded in the table, but for all specimens, the values conformed with the zero value or point of origin on the standard reference curve. Further conformity with the zero value was uniformly found in the determinations on the urine specimens taken from the treated groups prior to drug administration.

ARSENIC DETERMINATIONS ON THE DOG

Excretion Following Intravenous Administration.—Three infected dogs undergoing fifteen-day courses of intravenous therapy with arsenamide were also utilized for the determination of the excretion of arsenic in the urine and feces during the entire course of injections. The daily arsenic elimination observed in 1 of these dogs (No. 20) is shown in table 3. The urinary excretion of arsenic for the two-hour period immediately following the first injection accounted for two thirds of the total urinary excretion for the first day,

TABLE 2—Excretion of Arsenic in the Urine of Rats Following Single Doses of Phenyl Arsenoxides. Group Study of 3 Rats per Group, with Oral Doses Approximating 10 mg. of As per Kilogram and Intravenous Doses Approximating 2 mg. of As per Kilogram

Experiment (No.)*	Total dose (Mg. of As)	Mg. of As excreted in intervals (hours) after dose				Total		
		0-8	8-24	24-48	48-72	Mg.	Mg. As/kg.	% of dose
Mapharsen								
1	7.155	.218	.128	.132	.070	.548	.689	7.7
2	10.190	.035	.223	.154	.109	.521	.494	5.1
3	4.650**	.036	.081	.101	...	.218	.458	4.7
4	.990	.117	.048	.013	...	.178	.348	18.0
Arsenamide								
1	6.809	.147	.086	.083	.037	.353	.461	5.2
2	3.987**	.217	.082	.072	.031	.402	.918	10.1
3	5.251	.101	.066	.050	...	.217	.352	4.1
4	.961	.290	.016	.033	...	.339	.627	36.3
Carbarsone oxide								
2	8.349	.086	.110	.075	.052	.323	.348	3.9
3	6.402	.080	.053	.039	...	.172	.266	2.7
4	1.403	.216	.021	.000	...	.237	.333	16.9

*Experiments 1, 2, and 3, oral administration; experiment 4, intravenous administration.
**Two rats per group.

TABLE 3—Excretion of Arsenic in Urine and Feces of Dirofilaria Immitis-Infected Dog (No. 20) During Fifteen-Day Course of Intravenous Therapy with Arsenamide. Total Daily Dose, 6.0 mg. of Arsenic (.45 mg. of As per kilogram)

Day	Urinary arsenic		Fecal arsenic	
	mg.	% of dose*	mg.	% of dose*
2 (hr.)	.713	11.9		No feces
5 (hr.)	.114	13.8		No feces
24 (hr.)	.199	17.1	2.027	33.8
2	1.178	18.4	1.524	29.6
3***	1.183	19.7	3.928	43.5
4	1.530	21.2	2.758	44.1
5	1.886	23.3	3.552	47.2
6	1.863	24.6	1.330	43.0
7	1.501	24.7	3.606	45.4
8	2.028	25.8	2.034	44.0
9	2.090	26.9	3.568	45.7
10	1.850	27.3	3.858	47.6
11	1.917	27.7	3.539	48.7
12	2.002	28.2	5.244	51.9
13	2.195	28.8	4.936	54.3
14	1.992	29.1		No feces
15	1.953	29.4	2.968	50.3
16	.670	30.1	5.349**	56.3
Total	26.864	30.1	50.221	56.3

*Cumulative per cent of cumulative dose. **Includes intestinal contents. ***Dose = 5.2 mg. of As.

which was 17 per cent of the total dose of arsenic. During the course of the injections, the proportionate recovery of arsenic both in the urine and feces progressively increased until the total recovery in the urine and feces, respectively, was nearly twice that of the first day. Nearly twice as much arsenic appeared in the feces as compared with the total recovery of arsenic in the urine. Over 85 per cent of the total dose of arsenamide, in terms of arsenic, was accounted for in the urine and feces when the dog was destroyed forty-eight hours after the last dose was injected.

The studies on the remaining infected dogs receiving intravenous treatment with arsenamide revealed a pattern of excretion comparable to the foregoing and are not presented in detail, rather only the total urinary and fecal excretions of arsenic are summarized (table 4). This summary table includes not only the data on the 3 intravenously treated dogs but also the similar determinations on dogs receiving the arsenamide orally, thereby permitting a ready comparative evaluation of the two routes of administration. Of particular interest in this comparison is the total excretion of arsenic in the urine expressed on a unit weight (kilogram) basis, which will be referred to as the rate of excretion. The basic excretion rate was on the order

TABLE 4—Total Excretion of Arsenic in the Urine and Feces of Dogs During Courses of 15 Daily Doses of Arsenamide. With Two Exceptions, the Dogs Were Infected with Heartworm (Dirofilaria Immitis)

Dog (No.)	Weight, ave. (kg.)	Dose rate (mg. of As/kg.)	Total dose (mg. of As)	Total urinary arsenic			Total fecal arsenic	
				mg.	% of total dose	mg./kg.	mg.	% of total dose
Intravenous								
19	10.75	.45	69.420	16.819	24.2	1.565	42.349	61.0
20	12.25	.45	89.200	26.864	30.1	2.192	50.221	56.3
21	10.75	.23	36.000	11.434	31.8	1.064	19.905	55.3
Oral (in daily meal)								
10 N	9.0	4, later 2*	288.686	24.090	8.3	2.677	234.434	81.2
11 N	7.75	1.0	114.824	4.276	3.7	.552	69.528	60.6
12 b	7.6	1.0	104.991	4.030	3.8	.530	45.187	43.0
15	16.5	2.0	495.000	31.877	6.4	1.933	251.164	50.7
Oral (in gelatin capsules)								
22	7.0	4.0	420.000**	32.307	7.7	4.615	.....	...
Oral (in enteric-coated capsules)								
23	12.5	4.0	750.000	28.995	3.9	2.320	.....	...

*See table 5 for daily doses. N= normal dogs, *i.e.*, not infected with heartworm (*Dirofilaria immitis*). **Uncorrected for losses due to vomiting.

of 2 mg. of As per kilogram of body weight with the intravenous doses of .45 mg. of As per kilogram (dogs 19 and 20), and 1 mg. of As per kilogram with doses of .23 mg. of As per kilogram (dog 21). These values for the intravenous courses are regarded as an index of the amount of drug in the body and as the basic rates for comparing the values observed in the following oral administration trials.

Excretion Following Oral Administration.—Before comparable therapeutic activity could be expected in the oral administration trials, the rate of arsenic excretion in the urine as an index of the amount of drug present in the blood for therapeutic activity would have to approximate the basic excretion rate of the intravenous courses. Of the dogs receiving oral courses with arsenamide mixed with the daily meal for administration, 4 were utilized for the arsenic determinations (table 4). Two of these dogs, 1 infected (No. 15) and the other (No. 10 N) uninfected with heartworms, had rates of arsenic excretion in the urine approximating and exceeding, respectively, the basic rate of about 2 mg. of As per kilogram associated with intravenous courses at doses of .45 mg. of As per kilogram. The oral doses of these dogs were approximately 2 mg. of As per kilogram. Oral doses of 1 mg. of As per kilogram for the remaining normal (No. 11 N) and infected (No. 12 b) dogs in the group resulted in a rate of arsenic excretion in the urine which was only one fourth of the basic rate following intravenous doses of .45 mg. of As per kilogram.

The incorporation of the arsenamide powder in gelatin capsules and increasing the dose rate to 4 mg. of As per kilogram for an infected dog (No. 22) resulted in a rate of arsenic excretion of over 4.6 mg. of As per kilogram, which was well over twice the basic rate observed with intravenous doses of .45 mg. of As per kilogram. An additional arsenic excretion study on an infected dog (No. 23) undergoing a course of treatment with enteric-coated capsules containing the arsenamide powder at daily doses of 4 mg. As per kilogram also revealed a rate of arsenic excretion in the urine greater than the basic rate following intravenous doses of .45 mg. of As per kilogram. The excretion rate of this dog was only one-half that recorded for dog 22, receiving the arsenamide in gelatin capsules, although the dose rates were the same.

To indicate the general pattern of excretion which was observed during the prolonged oral administration trials, the daily urinary and fecal excretion of arsenic, which was determined on a normal dog (No. 10) during a course of administration of 15 daily doses of arsenamide powder mixed with the daily meals, is presented in table 5. The excretion of arsenic in the urine, expressed as the cumulative proportion of the cumulative dose, was not only maintained throughout the course but, as the treatment continued, there was a tendency for the proportionate recovery of arsenic in the urine to increase. The increase in this dog did not continue beyond the ninth day, and there was even a decrease thereafter; however, with the remaining animals, the increase in the excretion ratio continued throughout the course of treatment. Over 8 per cent of the total arsenic administered as arsenamide was recovered in the urine. The bulk

TABLE 5—Excretion of Arsenic in the Urine and Feces of a Normal Dog (No. 10 N) During a Course of 15 Daily Doses of Arsenamide Powder Mixed with the Daily Meals

Day	Dose (retained) (mg. of As)	Urinary arsenic		Fecal arsenic	
		mg.	% of dose*	mg.	% of dose*
1	40.000	2.284	5.7	12.134	30.3
2	40.000	3.268	6.9	No feces	
3	22.800	1.799	7.2	43.911	54.5
4	13.700	1.615	7.7	17.319	53.7
5	20.000	1.638	7.8	11.008	61.8
6	Refused to eat	1.452	8.8	6.972	66.9
7	20.000	1.421	8.6	18.130	70.0
8	Refused to eat	1.327	9.5	7.696	74.9
9	24.960	2.797	9.7	6.912	63.4
10	16.685	1.026	9.4	16.950	71.2
11	10.541	1.300	9.5	23.757	79.0
12	20.000	1.095	9.2	6.996	75.1
13	20.000	.871	8.8	32.718	82.2
14	20.000	.702	8.4	12.645	80.8
15	20.000	1.027	8.2	8.971	78.3
16	.....	.468	8.3	8.315**	81.2
Total	288.686	24.090	8.3	234.434	81.2

*Cumulative per cent of cumulative dose. **Includes intestinal contents.

of the arsenic was recovered in the feces during the course of administration, and the proportionate recovery in the feces of the cumulative dose of arsenic tended to increase throughout the course of treatment. Eighty-one per cent of the total dose of arsenic was accounted for in the feces of this dog when killed forty-eight hours after the last dose.

An additional study was made of the urinary and fecal excretion of arsenic following a single intragastric dose of arsenamide in a normal dog (table 6). The arsenamide was administered as a 1 per cent solution by stomach tube two hours after a preliminary meal, and the excretion was followed for the ensuing week. As previously found in the rat during oral administration, measurable quantities of arsenic were already present in the zero- to two-hour urine specimen. It will be noted that most of the arsenic that appeared in the urine was recovered in the first twenty-four hours after the dose, constituting over one half of the total urinary excretion, and that some arsenic was present in the urine through the sixth day. The first 24-hour recovery of arsenic (5.0%) compares closely with the observations on the first 24-hour urinary excretion of arsenic (5.7%) by the normal dog (No. 10) receiving a course of arsenamide mixed with the daily meals. In close agreement with the observations on the rat was the recovery of approximately 8 per cent of the oral dose of arsenic in the urine within the first seventy-two hours after the dose. The bulk of the oral dose of arsenic appeared in the feces, and more specifically, the most was recovered in the specimen voided by the dog on the third day. As with the urine, measurable quantities of arsenic were present in the feces through the sixth day.

Tissue Concentrations of Arsenic.—Further comparison between the oral and the intravenous routes of administration, as well as the three methods of oral administration, was obtained by the determination of the concentrations of arsenic in the tissues of the dogs on which the foregoing arsenic excretion studies were completed (table 7). With one exception, the determinations were made on wet tissues obtained at routine necropsy forty-eight hours after the last dose of the drug. Using the concentrations in the intravenously treated dogs (No. 19, 20, and 21) as the basis for comparison, it will be noted that both doses of .45 mg. of As per kilogram and .23 mg. of As per kilogram resulted in similar concentrations of arsenic in the tissues. Liver consistently contained the greatest concentration of arsenic, while intermediate concentrations were present in the bile, thyroid, adult filariae, and kidney. In the remaining tissues, the arsenic was more or less evenly distributed on a relatively lower order.

TABLE 6—Excretion of Arsenic by a Normal Dog Following a Single Intragastric Dose of a 1 Per Cent Solution of the Disodium Salt of Arsenamide. Total Dose of 17.8 mg. of As Given at the Rate of 1.78 mg. of As per kilogram

Time interval after dose	Urinary arsenic		Fecal arsenic	
	mg.	% of dose*	mg.	% of dose*
0-2 hours	.133	.7	No feces	
2-4 hours	.189	1.8	No feces	
4-6 hours	.125	2.5	No feces	
6-8 hours	.109	3.1	No feces	
8-24 hours	.346	5.1	No feces	
1-2 days	.252	6.5	.392	2.2
2-3 days	.308	8.2	10.920	63.6
3-4 days	.102	8.8	.524	66.5
4-5 days	.022	8.9	.143	67.3
5-6 days	.041	9.1	.032	67.5
6-7 days	.000	9.1	.000	67.5
Total	1.627	9.1	12.011	67.5

*Cumulative.

Following the oral courses of arsenamide powder mixed with the daily meals, the foregoing type of distribution, as well as a comparable order of concentration, were observed. Exceptions to the latter included dog 15 in which a slightly higher order was generally evident, and dog 12 b in which a consistently lower order was found. However, the necropsy and sampling of the tissues of the latter dog were made on the eighth day post-treatment, which was a delay of six additional days in comparison with the remaining dogs of the series. The oral courses of arsenamide powder incorporated both in gelatin capsules (dog 22) and in enteric-coated capsules (dog 23) resulted in the characteristic distribution pattern, but generally higher concentrations than those following intravenous administration were found. Exceptionally high concentrations were in the bile, spleen, and kidney of these dogs.

THERAPEUTIC ACTIVITY—ORAL ADMINISTRATION

As with the intravenous trials, attention was directed principally to the action on the adult heartworms in judging the effectiveness of the oral courses, while only pre-

treatment and post-treatment counts of the microfilariae were made. Fifteen daily doses were administered in all of the oral trials, because previous observations[9] on the intravenous use of arsenamide indicated that therapeutic effectiveness was dependent on daily injections for a period of two weeks. The therapeutic activity observed in the series of infected dogs receiving the arsenamide *per os* is recorded in table 8.

Adult Filariae.—A group of 6 infected dogs were fed the arsenamide powder by mixing it with the daily meals. The doses ranged from 0.5 to 4.0 mg. of As per kilogram of body weight. One dog (No. 12 b) of this group presented only fragments of dead worms at necropsy, while the remaining dogs presented only living worms. It is interesting to note the comparatively low dose (1 mg. As/kg.) received by this dog from which only fragments were recovered, and the low rate of arsenic excretion in the urine (*see* table 4), which was only one-fourth the basic rate with intravenous doses of .45 mg. of As per kilogram. Of further interest in connection with this dog was the delay of the necropsy until the eighth day post-treatment. A similarly delayed necropsy in another dog (No. 18) that received twice the dose rate resulted in the recovery of only living heartworms.

Increasing the doses to 4.0 mg. of As per kilogram and incorporating the arsenamide powder in gelatin capsules was tried in a single infected dog (No. 22). The purpose of this procedure was to study the effect of concentrating the dose in contrast with the foregoing trials in which the doses were distributed in a relatively large amount of food material for administration. Only living worms were recovered at necropsy, although the rate of arsenic excretion in the urine (*see* table 4) was the highest of any of the oral trials that were studied and well over twice the basic rate with therapeutically effective intravenous doses of .45 mg. of As per kilogram.

A limited supply of enteric-coated capsules containing arsenamide powder permitted only two trials on infected dogs (No. 23 and 24). In these trials, only the higher doses of 4 and 8 mg. of As per kilogram were given, with the latter being the maximum dose employed in the oral trials. The objective of using the coated capsules was to by-pass possible deleterious influences experienced by the drug in the stomach in the previous trials. The lower dose was associated with the recovery at postmortem examination of a single dead female worm and 36 living worms, while the higher dose resulted in the recovery of only living worms.

Microfilariae.—The microfilariae were not eliminated from the peripheral blood during any of the foregoing series of oral trials with arsenamide (table 8). With the exception of 2 dogs (No. 12 b and 15) in which the post-treatment count was on the order of one-half the pretreatment count, there was a general tendency for the number of microfilariae to increase during

TABLE 7—Concentration of Arsenic in Wet Tissues of Dogs Forty-Eight Hours After the Last of 15 Daily Doses of Arsenamide. With Two Exceptions, Dogs Infected with Heartworm (Dirofilaria Immitis)

Dog (No.)	Dose rate Mg. of (As/kg.)	Micrograms of arsenic per gram of wet tissue									
		Bile	Thyroid	Adult filariae	Lung	Spleen	Liver	Kidney	Heart	Muscle	Brain
Intravenous											
19	.45	1.7	1.7	1.0	.7	.8	5.2	1.4	<.2	.2	<.2
20	.45	3.5	3.3	4.0	.7	.7	6.7	2.2	.2	.3	.3
21	.23	1.7	1.1	1.2	.5	.8	5.5	2.5	.3	.2	..
Oral (in daily meal)											
10 N	4.0, 2.0*	2.1	1.4	...	.6	.5	5.5	1.8	.2	.2	.3
11 N	1.0	2.8	.8	...	.4	.6	7.5	1.8	.2	.2	.3
12 b**	1.0	1.5	.6	...	<.2	.2	2.0	.4	.3	..	..
15	2.0	9.0	.5	1.1	1.0	1.2	7.7	2.2	.5	.3	.5
Oral (in gelatin capsules)											
22	4.0	8.4	1.9	2.7	.5	3.0	7.7	10.8	.8	..	..
Oral (in enteric-coated capsules)											
23	4.0	12.2	2.0	1.3	.7	1.5	9.6	4.2	.6	.3	..

*For daily doses, *see* table 5. **Autopsy eight days after last dose, others at forty-eight hours. N = normal dogs, *i.e.*, not infected with heartworm (*Dirofilaria immitis*).

TABLE 8—Therapeutic Activity Against Dirofilaria Immitis of 15 Daily Dose Oral Courses of Arsenamide

Dog (No.)	Dose rate (mg. of As/kg.)	Time of autopsy	Microfilariae/cc. of blood		Status of adult filariae		
			Pre-treatment	Post-treatment	Living		Dead
					Male	Female	
	In daily meal						
13	0.5	48 hours	36,900	48,200	4	9	0
12 b*	1.0	8 days	46,800	19,900	0	0	fragments
18	2.0	8 days	9,700	21,600	25	26	0
14	1.0	48 hours	17,300	22,400	6	4	0
15*	2.0	48 hours	87,100	44,400	3	10	0
17	4.0	48 hours	26,300	32,600	8	12	0
	In gelatine capsules						
22*	4.0	48 hours	1,100	3,200	3	2	0
	In enteric-coated capsules						
23*	4.0	48 hours	15,600	33,400	22	14	1 female
24	8.0	48 hours	11,600	14,000	2	2	0

*Arsenic studies conducted on these dogs.

treatment. This tendency ranged from only a slight increase to a threefold rise.

Reactions Following Oral Administration.—The oral administration of arsenamide was in general well tolerated by the dogs (table 9). In none of the dogs was it necessary to terminate, or even temporarily suspend, the course short of the fifteen days, and in only 1 dog (No. 10 N) was the adopted dose rate reduced during the course of treatment (*see* table 5). This experience came in a preliminary trial on a normal dog and was not repeated in the therapeutic trials on infected dogs. Within the range of doses employed, the symptoms of intolerance to arsenamide bore no direct relation to the dose rate. Loss of appetite was the most common symptom of intol-

TABLE 9—Reactions Observed in Dogs During Oral Administration of 15 Daily Doses of Arsenamide. With Three Exceptions, the Dogs Were Infected with Heartworm (Dirofilaria Immitis)

Method of administration:	Mixed with food									In gelatin capsules	In enteric-coated capsules	
Dog (No.)	13	11 N	12 b	14	9 N	15	18	10 N	17	22	23	24
Dose rate (mg. of As/kg.)	0.5	1.0	1.0	1.0	2.0	2.0	2.0	4.0, 2.0	4.0	4.0	4.0	8.0
Day	Reactions											
1	....	C, V	....	....	....	V	....	D	....	V	....	....
2	....	C	....	....	....	....	....	....	....	....	....	....
3	....	....	....	....	....	....	....	C	....	V	....	....
4	D	....	....	....	....	....	....	C, V	....	....	....	....
5	D	....	V	....	....	V	....	....	....	....	....	....
6	....	....	....	....	....	....	....	C	....	....	....	....
7	....	....	....	....	....	....	....	....	....	....	....	D
8	....	....	V	....	....	....	....	C	....	....	....	....
9	....	....	....	....	....	....	....	C	....	....	....	D
10	....	....	....	....	....	....	....	C	....	....	....	D
11	....	....	C	....	....	....	....	C	....	....	....	D
12	....	....	C	....	....	....	....	....	....	C, V	....	....
13	....	....	V	....	....	....	....	....	....	C, V	....	....
14	....	....	C	....	....	....	....	....	....	C, V	....	....
15	....	....	C	....	....	....	....	....	....	C	....	....
16	....	....	C	....	....	....	....	C	....	C	....	....

D = loose stool; C = uneaten food; V = vomitus; N = normal dog, others infected with heartworm (*Dirofilaria immitis*).

erance associated with administering the drug mixed with the food. However, it is questionable whether the failure to eat was due entirely to a loss of appetite or in part, at least, because of the garlic-like odor imparted to the food by the arsenamide. The so-called loss of appetite was not complete, except on rare occasions, in that a part of the meal was ordinarily consumed. Thus, the adopted dose schedules were essentially administered successfully. Additional nominal losses were incurred in the adopted dose rates by the occasional episodes of vomiting which were in general associated with the loss of appetite. Characteristically, the vomiting was limited to a single act on a given day, as the nausea seemed transient. Loose stools were noted on rare occasions in the trials with mixing the arsenamide with the daily meals. This was transient and not associated with the foregoing symptoms. Failure to have a daily bowel movement was also observed occasionally in the dogs; however, it should be pointed out that the dogs had relatively little exercise. The single trial with administering arsenamide in gelatin capsules was accompanied by several episodes of vomiting and an almost complete loss of appetite near the end of treatment. In the two trials with administering arsenamide in enteric-coated capsules, only a transient period in which the stools were loose was noted midway during treatment of the dog receiving the higher doses.

ARSENIC DETERMINATIONS ON ADULT FILARIAE

Both living and dead heartworms that were recovered after therapy from certain of the infected dogs were analyzed for arsenic to compare therapeutic activity with the concentration of arsenic in the worms (table 10). This study included worms recovered from both dogs that had undergone oral and those that had undergone intravenous therapy with arsenamide. With one exception, the determinations were made on worms removed from the dogs forty-eight hours after the last dose of the drug. Dead worms only were recovered from 1 dog (No. 20) in this series, and these worms were found to contain the greatest concentration of arsenic (4 μg./Gm.). The arsenic concentration in the living worms recovered from the remaining dogs, including both oral and intravenous courses of treatment, were of a reduced order. These concentrations tended to be just above the 1-μg. level, with one exception (dog 22), in which the concentration (2.7 μg./Gm.) approached that of the dead filariae from dog 20. It was not possible to extend these observations to include additional dead worms, which would have been desirable in view of the fact that the crux of the comparison is based on a single determination (dog 20) in which the analyzed worms weighed only 0.5 Gm. The fragments of worms recovered from the orally treated dog (No. 12 b, tables 4, 7, and 8), in which only dead worms were observed, were not analyzed for arsenic because of the extremely low aggregate weight of the fragments. Dog 18, in which the relatively low concentration of arsenic in the worms was recorded, was the only dog in this series whose necropsy was delayed until the eighth day post-treatment rather than the routine forty-eight hours for the remainder.

LEUKOCYTE COUNTS

In the course of the investigations, a limited study was made on the leukocyte counts of certain of the infected dogs undergoing treatment with arsenamide. The

TABLE 10—Concentration of Arsenic in Adult Filariae, Living and Dead, Recovered from Infected Dogs After the Indicated 15 Daily Dose Courses of Treatment (with Arsenamide)

Dog (No.)	Route of administration	Dose rate (mg. of As/kg.)	Fate of adult filariae	Adult filariae	
				Weight*	Micrograms of As/Gm.
15	oral, in food	2.0	13 living	2.20	1.1
17	oral, in food	4.0	20 living	3.15	1.1
18	oral, in food	2.0	51 living	7.20	.2**
22	oral, gelatine capsules	4.0	5 living	0.55	2.7
23	oral, enteric-coated capsules	4.0	36 living 1 dead	3.45	1.3
19	intravenous	.45	6 living	0.75	1.0
20	intravenous	.45	6 dead	0.50	4.0
21	intravenous	.23	39 living	2.70	1.2

*Total weight analyzed in grams. **Autopsy eight days post-treatment, others forty-eight hours.

counts were followed both in the orally treated dog and the intravenously treated dog (table 11). The heartworms were killed in one (No. 20) of the dogs receiving the arsenamide intravenously, while only living worms were recovered from the remaining dogs. In none of the dogs, was the leukocyte count observed to be essentially changed during the course of arsenamide administration. The immediate posttreatment counts with both routes of administration and irrespective of the fate of the heartworms were essentially the same as the number recorded before treatment, while little deviation was noted in the periodic counts during the course of therapy.

In connection with the foregoing observations on the leukocyte counts of the dogs during arsenic therapy, a desire developed to study the leukocyte response to antimony. This comparison appeared desirable in view of the previous observations[10] that a marked leukocytosis occurred in heartworm-infected dogs during the intramuscular administration of the trivalent antimonial, monosodium antimony thioglycollate. In particular, the leukocytosis was associated with the elimination of the microfilariae from the peripheral blood without the death of the adult filariae; however, it was questioned whether the response was due entirely to the elimination of the microfilariae, or due in part, at least, to the influence of the metallic antimony *per se*. To ascertain the latter possibility, a series of daily intramuscular injections of monosodium antimony thioglycollate was given to a normal dog (No. A-1, table 11 and chart 1). The initial dose rate (1 mg.Sb/kg.) was accompanied by only a moderate increase in the number of leukocytes, but increasing the dose rate to 1.5 mg. of Sb per kilogram of body weight on the eighth day induced a more marked response. Severe local reactions accompanied the higher doses and general toxic manifestations were evident after the fourteenth day. The dose was again increased on the nineteenth day to 2 mg. of Sb/per kilogram and promptly resulted in the maximum leukocyte response, which constituted a fivefold increase over the pretreatment count. A rapid return to the pretreatment level resulted by the fourth day after the last of the injections.

DISCUSSION

This study was undertaken to develop, if possible, a procedure which would permit the oral administration of arsenamide in the treatment of canine filariasis. However, the results indicate that the oral route is not feasible, since the therapeutic trials uniformly failed to produce lethal action against the adult filariae.

The failure to obtain therapeutic activity after the oral administration of arsenamide can not be ascribed to a lack of absorption, since evidence has been presented to show that the extent of intestinal absorption provides sufficient arsenic in the system for possible therapeutic action. This evidence was gained through the studies which were conducted on the comparative urinary excretions and tissue concentrations of arsenic following both oral and intravenous administration. In the dog, oral doses of arsenamide as low as 2 mg. of As per kilogram of body weight daily resulted in a rate of arsenic excretion in the urine which approximated or exceeded the basic excretion rate following the therapeutically effective intravenous doses of .45 mg. of As per kilogram daily. Comparable excretion rates at this ratio of doses implies an

TABLE 11—Leukocyte Counts Observed on Dogs During Courses of Arsenamide and Monosodium Antimony Thioglycollate

Dog (No.)	Drug	Route	Dose rate	No. daily doses	Leukocytes per cmm.		
					Pretreatment	Maximum during treatment	Posttreatment**
18	Arsenamide	oral	2.0 mg. of As/kg.	15	16,700	20,000	13,600
17	Arsenamide	oral	4.0 mg. of As/kg.	15	15,200	19,200	16,900
19	Arsenamide	i.v.†	.45 mg. of As/kg.	15	22,000	25,600	23,200
20	Arsenamide	i.v.	.45 mg. of As/kg.	15	17,500	20,400	20,400
A-1*	Monosodium antimony thioglycollate	i.m.†	1.0 mg. of Sb/kg.	7	7,000	13,500	12,000
			1.5 mg. of Sb/kg.	11	12,000	26,500	18,000
			2.0 mg. of Sb/kg.	2	18,000	38,800	7,300

*Normal dog, others were infected with *Dirofilaria immitis*. **Twenty-four hours after last dose, except for final count on dog A-1, which was at ninety-six hours. †i.v. = intravenous; i.m. = intramuscular.

intestinal absorption on the order of 20 per cent, or comparable total amounts of arsenic in the system following both routes of administration. The studies on the concentrations of arsenic in the tissues is further indicative of sufficient absorption following oral administration. Oral dosage schedules of arsenamide as low as 1 mg. of As per kilogram resulted in concentrations of arsenic in the tissues which compared closely with those following the therapeutically effective intravenous dosage schedules of .45 mg. of As per kilogram. Although oral administration of arsenamide apparently resulted in the absorption of sufficient arsenic, other factors may be held accountable for the lack of therapeutic activity.

Of primary importance in this consideration is the inherent limitation of the technique used in this study for the determination of the drug. The technique indicates only the arsenic content originally incorporated in the arsenamide molecule, and provides no information as to the integrity of the molecule at the time the arsenic fraction is recovered in a specimen. Although specific information on arsenamide is not available, studies on the related phenyl arsenoxide, mapharsen, have shown that arsenic determinations *per se* provide only limited information on the therapeutic potential of the compound. In this connection, Wright and Peters[16] reported the relatively short duration of the trypanocidal activity of mapharsen after intravenous administration to rats, although arsenic could be recovered from the blood and tissues for relatively long periods after the injection. More recently, Maren,[7] in running parallel determinations on arsenic and on o-aminophenol moiety after intravenous injection of mapharsen to dogs, found the o-aminophenol moiety disappeared rapidly from the plasma after three hours but the arsenic content remained fairly consistent through forty-eight hours. Without this latter type of information on arsenamide and with no present means for its determination, a more critical evaluation of the therapeutic potential of the orally or even the intravenously administered arsenamide can not be made. However, in view of the observations on mapharsen, it is reasonable to assume that a similar degradation and inactivation occurs to arsenamide in the body and that the determinations of arsenic *per se* are not necessarily an index of therapeutic potential. During the oral administration of arsenamide, the degradation and inactivation may occur even before or during absorption, as well as after. Thus, following oral administration, there is at least greater opportunity for the inactivation to take place before the drug

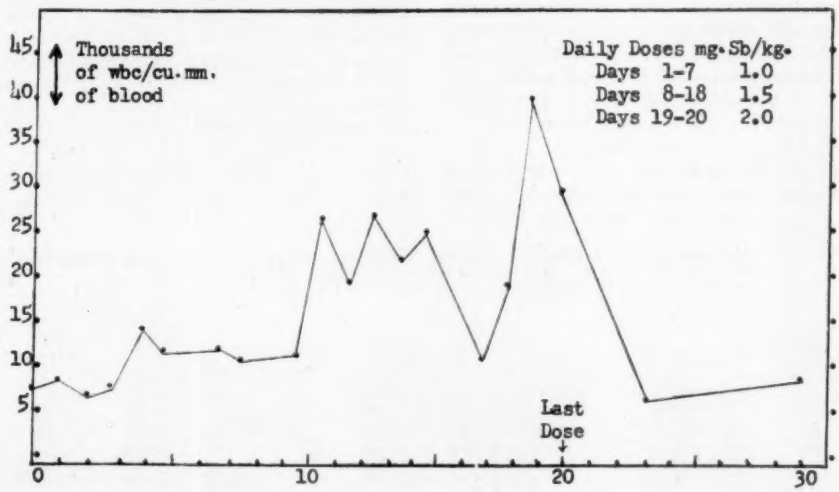

Chart 1—Leukocyte counts on a normal dog receiving daily intramuscular injections of monosodium antimony thioglycollate. Injections omitted on ninth and sixteenth days.

reaches the site of therapeutic activity or before sufficient concentrations of the therapeutically active drug are built up. Irrespective of the site of the inactivation, the failure of the active drug to reach the adult filariae in sufficient concentration could explain the lack of therapeutic action and render the arsenic determinations as uninformative and misleading.

Although no evidence was obtained to indicate the nature of the *in vivo* degradation of arsenamide that was postulated in the foregoing discussion, previous observations[11] on the stability of arsenamide solutions during storage have shown that oxidation may occur. When sealed in 5-cc. amber glass ampules and stored at either 5 C. or at room temperature, solutions of arsenamide appeared to be stable for nearly two years, or when the periodic pH determinations, iodine titrations, and toxicity determinations (acute lethal $dose_{50}$) on mice were terminated. The oxidation was detected at fifteen months by iodine titration in a solution that had been packaged in a 25-cc. membrane, rubber-stoppered amber bottle and stored at 5 C. In the present study, limited evidence was obtained which suggests that the therapeutic activity of arsenamide solutions may also be reduced upon storage. A single therapeutic trial of .45 mg. of As per kilogram daily for fifteen days on an infected dog with a solution of arsenamide that had been prepared eighteen months previously resulted in the recovery of only living heartworms at necropsy. This 1 per cent solution was packaged in a 60-cc. membrane, rubber-stoppered amber bottle, kept in the refrigerator at 5 C. during the eighteen-month storage period, and was untapped prior to its use on this dog. Similar dosage schedules of freshly prepared solutions of arsenamide in additional infected dogs uniformly resulted in the death of the worms, which does suggest that the eighteen-month storage period of the foregoing solution, even under apparently favorable conditions, had been detrimental to the arsenamide and had rendered it at least therapeutically less active.

One should not overlook the possibility that effective intravenous treatment of canine filariasis with arsenamide may be due to the relatively high blood concentration of drug which follows the injections that are repeated daily for fifteen days. Furthermore, it is unreasonable to assume that the order of doses employed in the oral administration trials resulted in blood concentrations comparable to the maximum concentrations immediately following the intravenous injections, although it was shown that intestinal absorption started shortly after oral administration and that over the course of treatment comparable total arsenic was present in the blood.

The arsenic determinations on the adult filariae presented a suggestive relationship which may bear on the difference between effective and noneffective treatment, either oral or intravenous. A single determination on dead filariae from an intravenously treated dog revealed a concentration of arsenic in excess of that determined in living worms from both orally and intravenously treated dogs. The latter concentrations (1.0 μg. As/Gm.) tended to be on the order of one-fourth the concentration (4.0 μg. As/Gm.) in the dead filariae, although one determination on living worms revealed a concentration (2.7 μg. As/Gm.) which approached that of the dead filariae. This marginal difference of concentrations can not be properly evaluated because the minimal *in vivo* arsenic concentration for lethal action on the heartworm is unknown. Furthermore, there is no evidence that arsenic content, *per se*, offers a measure of therapeutic effectiveness. However, it is of interest to note in the studies of Otto and Maren[11] that the arsenic concentrations in dead filariae from intravenously treated dogs was never less than 2 μg. of As per gram and ran as high as 5 μg. of As per gram. This range is not only in agreement with the present observation but conforms with the *in vitro* concentrations which killed the microfilariae of *D. immitis* and the adults of *Litomosoides carinii* in less than a day. Thus in comparison, the predominating 1-μg. concentrations in the living worms of the present study appear as a marginal concentration for both *in vivo* and *in vitro* activity, and the concentration of 2.7 μg. in the living worms from the 1 orally treated dog may well represent an extreme variation from the average. Nevertheless, the marginal difference of arsenic concentrations in the adult filariae do not appear to offer the entire explanation for effective or noneffective arsenamide treat-

ment. Daily intravenous doses of .23 and .45 mg. of As per kilogram, as well as daily oral doses ranging from 1.0 to 4.0 mg. of As per kilogram resulted in the same general order of arsenic concentrations in the hosts' tissues. In contrast, the arsenic content (4 μg./Gm.) of the dead worms from the dog receiving the intravenous doses of .45 mg. of As per kilogram was higher than the general concentrations in the hosts' tissues, whereas the arsenic content (1 μg./Gm.) of the living worms from the remaining animals, including both orally and intravenously treated dogs, was below the general concentrations in the hosts' tissues. This suggests that a difference of affinity of the arsenic for the worms was playing a role in differentiating between effective and noneffective treatment. No evidence was obtained to account for the affinity differences; however, it does appear to be reasonable that the postulated *in vivo* degradation of the arsenamide may, in turn, result in the loss of affinity. This would account for noneffective treatment, either oral or intravenous, in that insufficient concentrations of the active drug gains and maintains access to the worms, whereas effective therapy provides sufficient active drug at the site of action before the degradation and loss of affinity occurs.

The minimal effective intravenous dosage schedule of arsenamide was higher than that previously indicated by Otto and Maren.[9] They effected the death of the adult filariae of the dog at doses as low as .23 mg. of As per kilogram daily for fifteen days. Uniform lethal action against the adult worms was not obtained in the present study at doses below .45 mg. of As per kilogram daily for fifteen days. This is in support of the dosage schedule of arsenamide of daily injections of .45 mg. of As per kilogram for fifteen days that has been recommended for the practical therapy of heartworm infection. The results of the attempts to reduce the number of injections to ten for the intravenous therapy of canine filariasis indicate that such a procedure is not feasible. Although this schedule resulted in the death of some of the worms, a regularly lethal action was not produced even by increasing the daily dose rate to .60 mg. of As per kilogram for ten days.

The observation of living microfilariae *in utero* in dead female heartworms recovered from intravenously treated dogs is of more than passing concern in this discussion. Of particular interest was the active microfilariae persisting even in the dead female worms which were undergoing disintegration. This observation is in accord with the previously recognized selective action of arsenamide of failing to clear the blood of microfilariae during a course of treatment, although the adult heartworms are killed. Thus, the selective action of arsenamide in the dog appears consistent in failing to act on the microfilariae, either circulating or *in utero*, during a course of intravenous treatment, although possible selective action on earlier embryonic stages was not excluded.

Leukocyte counts on heartworm-infected dogs remained essentially unchanged during both oral and intravenous courses of arsenamide. The oral and one of the intravenous courses were therapeutically ineffective; however, the remaining intravenous course of .45 mg. of As per kilogram daily for fifteen days resulted in the recovery of only dead heartworms at autopsy. Thus, this limited study indicates that neither the arsenic nor the death of the adult worms induces a leukocytosis in dogs during a standard intravenous course of arsenamide.

On the contrary, monosodium antimony thioglycollate induced a marked leukocytosis in a normal dog during a series of intramuscular injections. Because of the route of administration and the resultant tissue damage, the leukocyte response may be ascribed to the antimony *per se*, to the tissue reaction, or both. However, this observation clarifies to some extent the factors which may contribute to the leukocytosis which was previously observed[10] during the treatment of heartworm-infected dogs with intramuscular injections of monosodium antimony thioglycollate. In the infected dogs, the simultaneous elimination of the microfilariae from the blood had to be considered as a factor in inducing the leukocytosis. The present study does not exclude the latter as a contributing factor, but the study does indicate that the elimination of the microfilariae is not the only factor, providing it is a factor, since the intramuscular injections of the antimonial *per se* can induce a leukocytosis. Although this study was lim-

ited, it carries the added implication that caution should be exercised in the interpretation of a leukocytosis occurring during the treatment of filariasis with a metallic compound.

Summary

The uniform failure to kill the heartworms in the oral trials indicates that the *per os* administration of arsenamide is not a feasible procedure for treating this infection. Therapeutic ineffectiveness could not be ascribed to insufficient enteric absorption, since it was found that adequate drug, in terms of arsenic, was available for therapeutic action during the oral trials at doses as low as 2 mg. of As per kilogram of body weight. To account for the absence of therapeutic activity in the presence of sufficient arsenic, it was postulated that degradation and inactivation of the arsenamide occurred before, during, or after enteric absorption. Furthermore, evidence was presented to the effect that the degradation was tied in with a loss of affinity of the drug for the heartworms as the essential determining factor of therapeutic activity.

In the intravenous trials, only daily doses of .45 mg. of As per kilogram for fifteen days regularly killed the heartforms. Attempts to reduce the number of injections or the dose rate sacrificed therapeutic effectiveness. Living microfilariae were found to persist *in utero* in dead and fragmenting female worms recovered from intravenously treated dogs. This observation is in accord with the already established characteristic action of arsenamide in the dog of failing to eliminate the microfilariae from the peripheral blood during treatment, although the heartworms are killed. Within the limits of the doses employed, neither the oral nor the intravenous administration of arsenamide was found to affect the leukocyte count of infected dogs during treatment, even in the intravenous trials in which the heartworms were killed. On the contrary, the intramuscular injection of the trivalent antimonial, monosodium antimony thioglycollate, in a normal dog produced a marked leukocyte response.

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Circulation of Blood Through the Canine Kidney

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LITERATURE CONCERNED with renal circulation contains a great deal of discord, not only with reference to the existence and course of minute vessels, but also with respect to the main vascular pathways. This paper, devoted exclusively to the renal circulation of the dog, deals only with those vessels under the most controversy.

Maceration techniques with neoprene latex and vinylite resin were used extensively. Experimental surgery was done to determine the effect on kidney circulation by stimulants, as well as to ascertain the results of surgically induced renal hypertrophy upon blood flow through the kidney.

LITERATURE

Classically, blood to the kidneys passes from the aorta through the renal artery, end arteries, interlobar, arcuate, and interlobular arteries, and then to glomeruli by means of afferent arterioles. Efferent arterioles from glomeruli go to the apexes of their renal pyramids. Stellate veins successively supply converging interlobular, arcuate, and interlobar venous vessels before the renal vein reaches the caudal vena cava.

Arcuate arteries, according to Smith and Copenhaver,[16] are the archings of the interlobar arteries between the medulla and the cortex and do not communicate with each other. Maximow and Bloom,[6] to the contrary, illustrate arcuate arteries as being in continual communication throughout the junction of the medullary and cortical areas.

Virchow[19] is of the opinion that the renal medulla receives blood primarily from direct nutrient branches of arcuate or interlobular arteries, called arteriolae rectae verae. Bowman[2] and Huber[3] deny their existence in the human kidney, while MacCallum[5] emphasizes that there are no arteriolae rectae verae in the dog.

Anatomists and physiologists, with the exception of Bowman[2] and Trueta,[18] agree that vasa afferentia are larger than vasa efferentia. Vasa afferentia, according to Smith and Copenhaver,[16] often redivide to supply a cluster of malpighian corpuscles.

Smith[15] and Oliver[11] state that Ludwig's arterioles (branches of vasa afferentia in the corticomedullary zone passing directly into the medullary peritubular capillaries) are not present in normal kidneys.

Smith,[14] McManus,[7] White,[20] Oberling,[10] and Richards and Schmidt[12] favor the theory of glomerular intermittence. Trueta *et al.*[18] not only suggest that glomeruli may become temporarily inactive, but state that blood reaching the kidney has two potential routes, juxtamedullary and outer cortical. Leiter[4] and Shannon[13] do not subscribe to glomerular intermittence.

Trueta *et al.*[18] state that vasa efferentia associated with juxtamedullary glomeruli are "much larger" in diameter than those connected to outer cortical glomeruli. According to Oliver,[11] efferent arterioles either break up directly into intertubular capillary networks or continue down and pass into the medulla as arteriolae rectae spuriae.

Arteriovenous anastomoses have been described between interlobar arteries and veins, between arcuate arteries and veins, and also between interlobular arteries and veins. Spanner[17] and Smith[14] found evidence of these anastomoses, whereas Oliver[11] did not. Leiter[4] discredits any functional significance of arteriovenous anastomoses in normal kidneys.

According to Trueta *et al.*,[18] under conditions of shock, the comparatively large vasa efferentia of the juxtamedullary glomeruli provide a by-pass through which arterial blood may be shunted into the renal veins without passing through the peripheral layers of the cortex. These authors state that this "fundamental difference" between outer cortical and juxtamedullary glomeruli is "clearly seen" in the kidneys of man, dog, and cat. Moyer and his associates[8] had negative results in demonstrating the existence of a juxtamedullary by-pass.

MATERIALS AND METHODS

A total of 67 fresh and 50 preserved kidneys were studied, from dogs of various ages, sizes, and breeds. The kidneys were excised and injected with neoprene latex or vinylite resin shortly after the dogs had died (due to causes other than renal disease). Injection pressures averaged 340 mm. Hg. for arteries and 200 mm. Hg. for veins.

The vinylite used was a 12.5 per cent solution

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of vinylite resin in acetone to which red, blue, and yellow pigments had been added. After injections were completed and the vessels ligated, each kidney was immersed in 37 per cent hydrochloric acid for maceration. After five days, the hardened cast was washed in a gentle, indirect flow of cold water.

Latex injections were executed in a manner similar to those done with vinylite. In general, arteries received from 3.0 to 7.5 cc., veins from 3.2 to 7.9 cc., and the ureter and pelvis, 3.0 to 7.6 cc. Latex casts were a more exact, though less rigid, replica of the renal vascular tree.

Vinylite never penetrated any farther than the afferent glomerular arterioles, whereas latex filled the glomerular capillaries and the efferent glomerular vessels.

Four dogs were used for the purpose of repeating Trueta's experiments[18] with epinephrine and India ink injections. Each dog was asymptomatic and in good physical condition. Pentobarbital sodium was given intravenously until surgical anesthesia was produced. The kidneys were exposed by high paracostal incisions. Higgins' American India Ink diluted with three parts of distilled water was used as the injection mass.

In 2 of the dogs, India ink solution was injected into the left renal artery and allowed to circulate through the kidney. When it was ascertained that the ink was coming through the renal vein, both the renal artery and renal vein were clamped off and the kidney removed. In both dogs, the right kidney was exposed in a manner similar to the left. Epinephrine (1: 1000) was given in the cephalic vein; 1 dog receiving 1/10 of 1 cc. and the other 1 cc. Immediately, the heart beat slowed and strengthened. Three seconds after the epinephrine injection, India ink was administered by way of the renal artery and the kidney was removed.

The third dog was injected with India ink into both renal arteries, whereas the fourth dog received ¼ of 1 cc. of epinephrine in the sublingual vein, followed by an injection of India ink into the left renal artery. The kidneys were removed after the injections. After thirty minutes, when the pulse of the fourth dog was back to normal, ¾ of 1 cc. of epinephrine was given intravenously, followed by the usual India ink injection, ligation, and excision of the right kidney. Distribution of pigment in the renal vessels was studied histologically.

To determine the effect that unilateral nephrectomy had upon the blood circulation of the remaining kidney, a 3-year-old Collie-Shepherd cross, in apparent good health, was utilized. Through a high paracostal incision, the left kidney was emptied of blood and injected with latex *in vivo*, then removed and macerated.

After five months, the right kidney was injected with latex and removed. When maceration was complete, both right and left renal circulatory casts were compared grossly and microscopically. Measurements were taken of many areas in both kidneys to compare the relative differences. In each kidney, juxtamedullary and outer cortical glomeruli were compared, as well as afferent and efferent arterioles of individual glomeruli. The basic vascularity of the "normal" kidney was compared to that of the "hypertrophied" kidney.

OBSERVATIONS

From the study of injected specimens of dog kidneys, it was noted that the renal

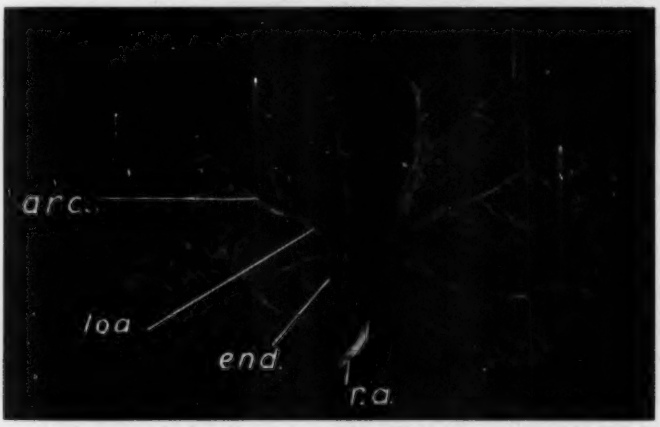

Fig. 1—Photograph of vinylite cast of renal arterial circulation. r.a.=renal artery; end.=end artery; loa.=interlobar artery; arc.=arcuate artery.

artery bifurcates into dorsal and ventral branches. The bifurcation usually takes place 1 cm. outside of the renal hilus. The two branches of the renal artery are "end branches," each of which divides into two to four interlobar arteries. At the corticomedullary junction, the interlobar arteries branch into arcuate arteries (fig. 1). These usually leave the interlobars at acute angles and radiate toward the periphery of the cortex, where they redivide into numerous interlobular arteries. Vasa afferentia leave these to supply the glomeruli and vasa efferentia. The venous circulation follows its retrograde path from the capsular stellate veins to the renal vein. Some efferent arterioles leave glomeruli and break up into a network of coiled arterioles, which supply the convoluted tubules of the cortex with blood.

In this study, the right renal artery was invariably longer than the left, whereas the left renal vein was longer than its counterpart of the right side. In 14 dogs, 17 of their 28 kidneys had renal arteries that did not bifurcate before entering the renal hilus (fig. 2). Of 117 kidneys, 29 had completely double renal arteries. Six renal arteries divided into five branches before entering the hilus, four arteries branched six times, and two arteries branched seven times. In one case of bilaterally double renal arteries in which the vessels left the aorta 1 cm. apart, the right cranial branch divided twice and the caudal three times before disappearing into the renal hilus. The left arteries subdivided into four and three, totaling seven vessels going into the left kidney.

In three vinylite specimens, Ludwig's arterioles were ascertained arising from afferent arterioles and descending directly to peritubular plexuses. Arcuate arteries were divergent branches of the interlobar arteries and, except in comparatively few cases which were from older dogs, did not have an arched appearance (fig. 1).

Venous arcuate vessels, on the other hand, united consistently to form elaborate arches. In most specimens, arcuate veins from each side spanned the area left vacant by medullary tissue, joining both halves of the kidney over the roof of the pelvis. When only the pelvis and renal vein were injected

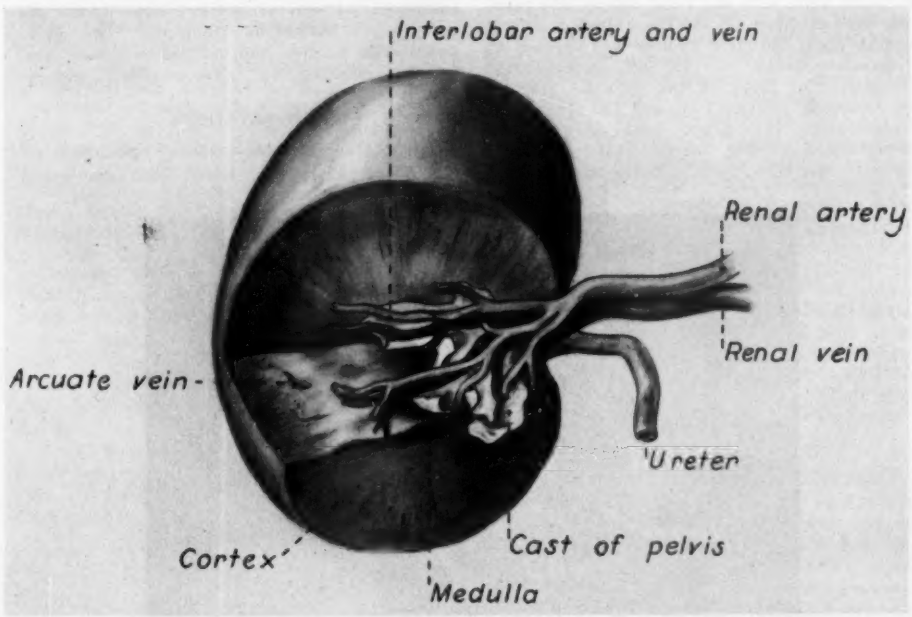

Fig. 2—Drawing of craniocaudal view of the right kidney of a dog. The parenchyma of the organ is cut in transverse planes, revealing the manner in which the arterial and venous branches engulf the renal pelvis. Vinylite was used as the injection mass, giving rigidity to the vessels and ureter.

with vinylite, the venous arches and the venae stellatae were clearly pronounced (fig. 3). Stellate veins were very numerous and widespread in the periphery of the dog kidney.

There was no evidence of arteriolae rectae verae in any of the specimens injected with vinylite but seven of them were found in latex casts. Arcuate arteries fed into interlobular arteries in all vinylite specimens without giving off tangent vessels. No arteriovenous anastomoses were found.

Dissection of latex specimens injected *in vivo* revealed that both outer cortical and juxtamedullary glomeruli were equally distributed and equally prominent (fig. 8, 9, 10, and 11).

Arteriolae rectae spuriae, running from juxtamedullary glomeruli into the medulla, were seen in a few instances. In two cases, afferent and efferent arterioles anastomosed with each other just outside their glomeruli.

In vivo injections of India ink and epinephrine gave consistent results. Kidneys that received only an ink injection showed an equal distribution of pigment. There was little discernible difference between outer cortical and juxtamedullary areas. Also, there was no appreciable difference in the amount of ink in the glomeruli of the epinephrine-injected dogs than in the dogs that received no epinephrine. Regardless of the dosage of the circulatory stimulant, the outer cortical parts of the kidneys were amply injected.

There were some striking contrasts between the two kidneys of the animal (dog 9) that had a left nephrectomy, following an *in vivo* renal artery injection of latex (fig. 4 and 5). The left kidney, upon removal, measured 2 9/16 in. in length, whereas the right, removed five months later, measured 3 2/16 in.

An unusual aspect of the hypertrophied specimen was the presence of coiled inter-

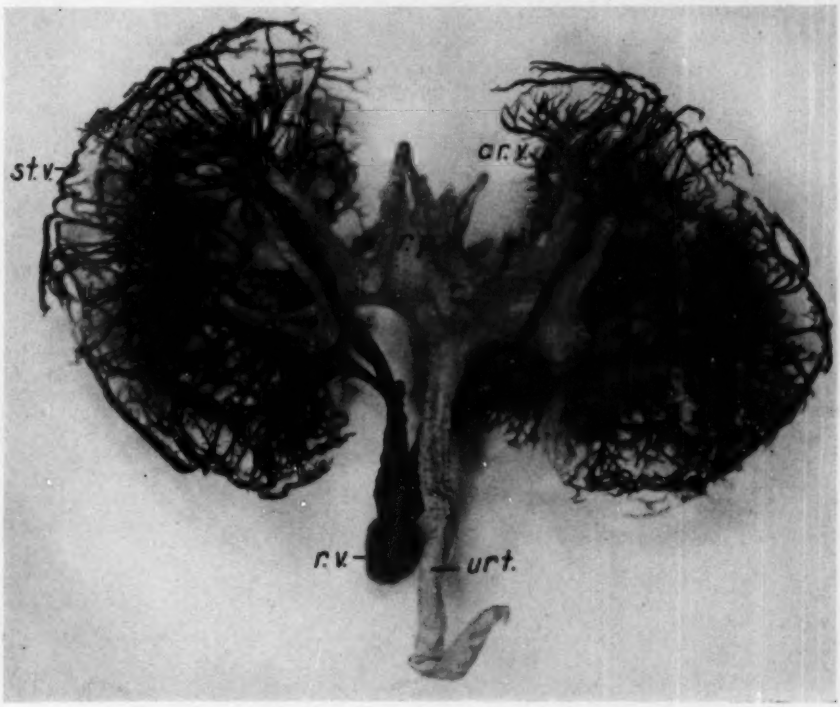

Fig. 3—Vinylite cast, showing renal pelvis and venous circulation of the dog kidney. The veins over the center of the renal pelvis have been dissected off to illustrate the venous relationship to it. ar.v.=arcuate vein; st.v.=stellate vein; r.p.=renal pelvis; urt.=ureter; r.v.=renal vein.

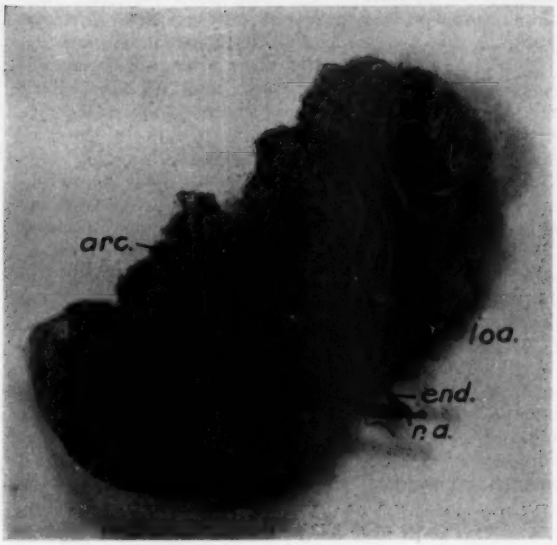

Fig. 4—Latex cast of arterial circulation of normal left kidney from dog 9. r.a.=renal artery; end.=end artery; loa.=interlobar artery; arc.=arcuate artery.

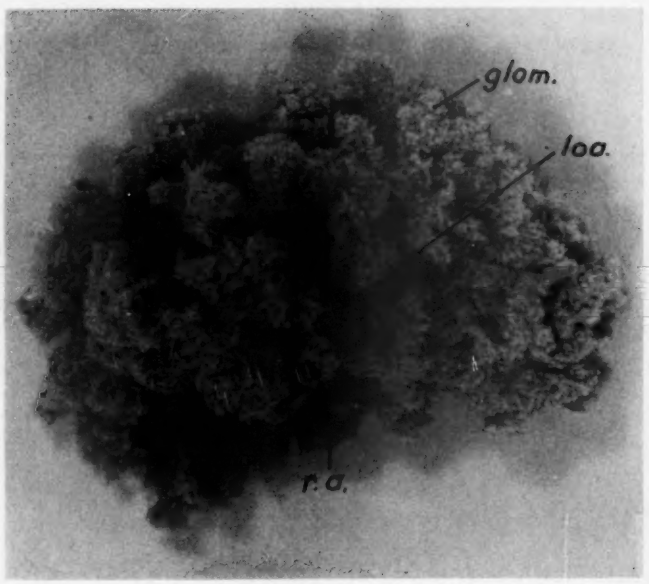

Fig. 5—Latex cast of hypertrophied right kidney from dog 9. This kidney was injected and removed five months after the left one. r.a.=renal artery; loa.=interlobar artery; glom.=glomeruli.

arterial vessels of fairly large caliber, forming a complex network. In this specimen, an arteriole left an interlobar artery, coiled in a regular pattern and, after making a loop, joined the arcuate artery branching off the interlobar artery of its origin (fig. 6 and 7).

For the purpose of determining the effect of hypertrophy upon the circulation of a dog kidney, measurements of vascular structures were taken from the kidneys of the aforementioned nephrectomized dog 9. In order to check Trueta's differentiation of outer cortical and juxtamedullary glomeruli, diameters of their efferent arterioles were compared over a series of latex casts from normal kidneys. Since perusal of the literature also brought to light a difference of opinion with regard to the size of afferent versus efferent glomerular arterioles, measurements were made of a large number of these (table 1).

These data show the absence of statistically significant differences between outer cortical and juxtamedullary arterioles within each kidney. However, the data for the

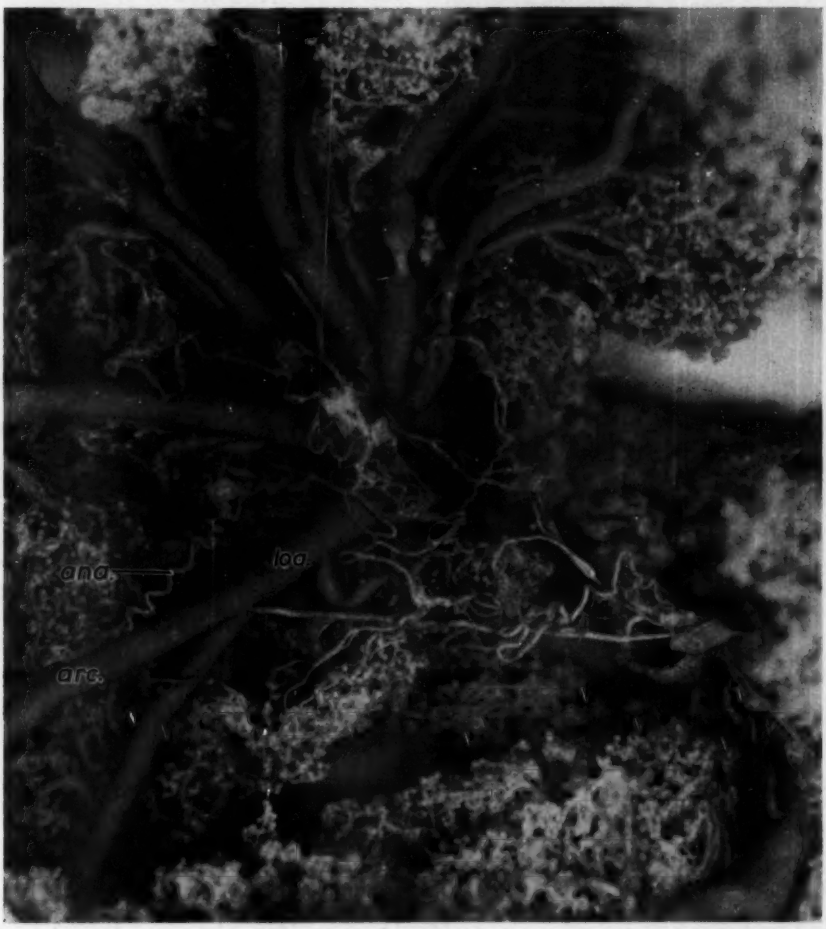

Fig. 6—Photograph of latex arterial cast from hypertrophied kidney. The vascular network in the renal hilus is apparent. Note the artery-to-artery anastomosis as well as the vascular constrictions. arc.=arcuate artery; loa.=interlobar artery; ana.=anastomotic artery.

hypertrophied right kidney of dog 9 show a difference between the diameters of outer cortical and juxtamedullary glomeruli amounting to 15.4 μ, with a standard error

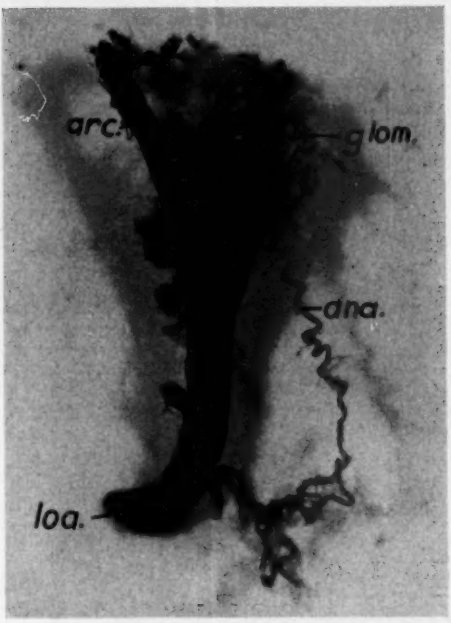

Fig. 7—Dissected and enlarged portion of cast in fig. 6. The arterial anastomosis leaves the interlobar artery, descends downward and to the right, then ascends in a coiled manner to its neighboring arcuate artery. loa.=interlobar artery; arc.=arcuate artery; ana.=anastomosis; glom.=glomerular clump.

of 6.8. Thus a "t" (measure of reliability) of 2.26 indicates that the deviation is great enough not to be due to chance alone.

Of 360 individual measurements of afferent and efferent arterioles, the afferent artery of a glomerulus was larger in diameter than the efferent of the same glomerulus, in every case but two. Outer cortical vasa efferentia were usually larger than juxtamedullary vasa efferentia.

In comparing measurements from the two kidneys of dog 9, glomeruli of the normal left kidney were decidedly smaller than those of the hypertrophied right kidney. Individual glomeruli were increased in size, as was the entire right kidney. Figures 8, 9, 10, and 11 compare glomeruli from the normal left kidney, along with their vessels, with those from the hypertrophied organ. Constrictions occurred in many of the finer vessels and some of the larger vessels (fig. 6).

DISCUSSION

Contrary to the majority of authors who describe arcuate arteries as running parallel to the surface of the kidney, between cortical and medullary zones, those seen in this study separated at acute angles from interlobar arteries and divided into interlobular vessels. Morison's opinion[9] that they assume their fully arched appearance only as age advances may explain their almost complete absence in the younger dog. Only a few were seen that fitted the typical histology text description, and these were from dogs ranging from 7 to 9 years of age. No arcuate arteries, arched or otherwise, connected throughout the kidney in a continuous arterial arcade.

When epinephrine was administered prior to India ink injections into the renal artery, there should have been a distinct and marked flow of circulation away from the cortex and toward the medulla, according to Trueta *et al.*[18] This phenomenon is one of the main justifications for their theory of a double renal circulation. Hemorrhage, shock, etc., releasing epinephrine into the circulation, are supposed to cause this by-pass to function in the normal animal. Neither gross nor microscopic examination of the kidneys confirmed Trueta's

TABLE I—Diameters of Vessels and Glomeruli from Dog Kidneys

Renal vascular elements	Dog 11 9-yr.-old 57 lb. Mean and s.e.* (μ)	Dog 9 3-yr.-old 35 lb. (left kidney) Mean and s.e. (μ)	Dog 9 (hypertrophied right kidney) Mean and s.e. (μ)
Outer cortical glomeruli	232.0±6.9	172.3±2.9	238.7±5.2
Juxtamedullary glomeruli	234.7±4.2	167.3±4.2	223.3±4.4
Outer cortical vasa afferentia	48.7±2.5	35.0±1.2	42.0±2.0
Juxtamedullary vasa afferentia	46.3±2.6	32.0±1.9	42.0±2.0
Outer cortical vasa efferentia	31.7±2.1	18.7±1.3	26.0±1.3
Juxtamedullary vasa efferentia	28.3±1.7	16.7±1.3	25.3±1.3

*Standard error.

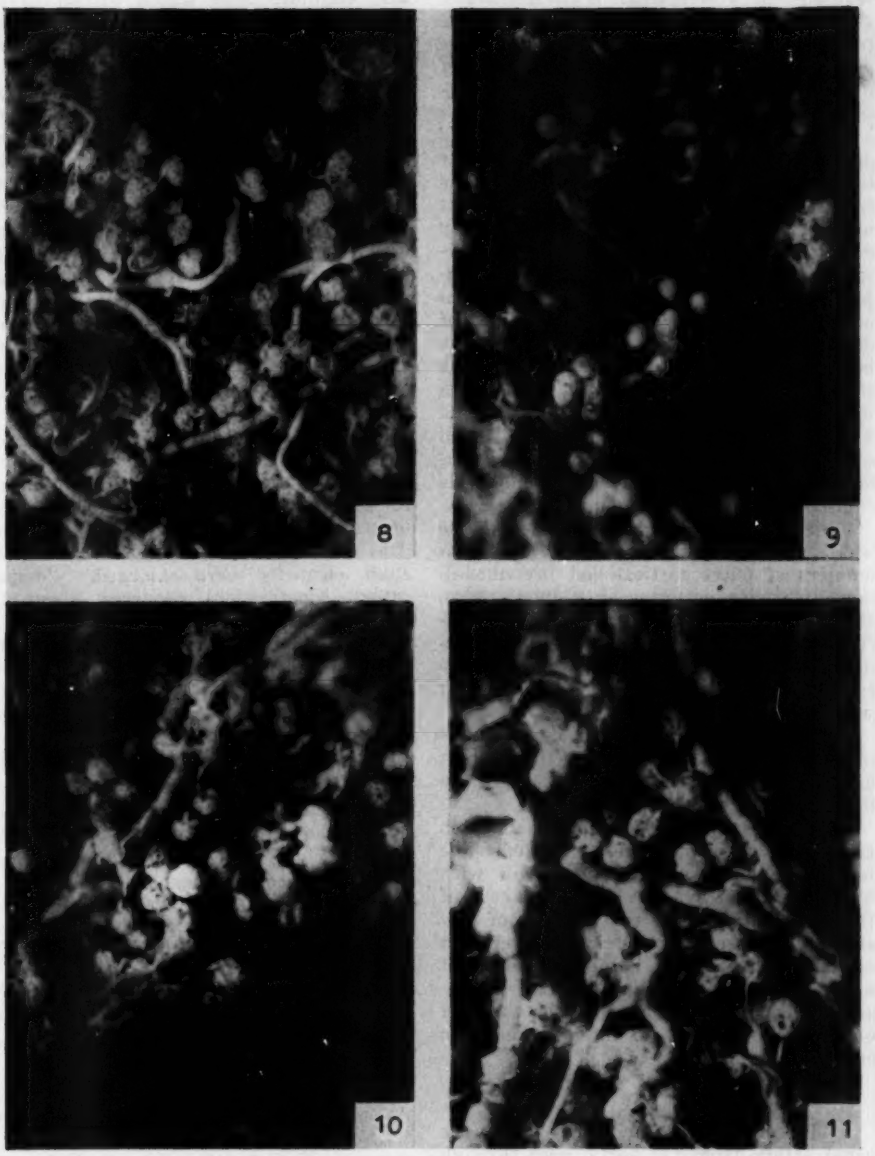

Fig. 8—Microscopic view of latex-injected outer cortical glomeruli with their afferent and efferent arterioles. Normal left kidney of dog 9. x 25.

Fig. 9—Juxtamedullary glomeruli and vessels from same kidney as in fig. 8. x 25.

Fig. 10—Injected glomeruli and arterioles from outer cortical region of hypertrophied dog kidney. x 25.

Fig. 11—Glomeruli and vessels from juxtamedullary area of same kidney as in fig. 10. See table 1 for comparative measurements of glomeruli and arterioles from normal and hypertrophied kidneys. x 25.

results. In a few small scattered areas, glomeruli were unfilled. This may be due to intermittent function on the part of the glomeruli, but more likely is the result of mechanical failure of the pigment to penetrate every vessel.

Trueta *et al.*[18] claim to be the first to describe coiled interarterial vessels similar to those found in this investigation and, as far as can be determined, up to present writing are the only ones to do so. They depict them as arising from interlobar arteries, either those of their origin or adjacent ones, which they join at some peripheral point. Spanner[17] gave a drawing of one of these vessels, but described it as being part of an arteriovenous shunt. In this case, it was a communication between interlobar and arcuate arteries. Trueta and his co-workers theorize that these arterial connections may be concerned with the regulation of blood pressure to the fragile capillary network of the mucosa.

Contrary to their findings, however, there was no significant disparity between diameters of outer cortical and juxtamedullary arterioles within each kidney. Also, their statement that the caliber of the efferent vessel of a juxtamedullary glomerulus greatly exceeds that of an outer cortical efferent arteriole is in direct conflict with the data in table 1.

This work agrees with the observations of Bensley and Bensley[1] in that the afferent arteriole of a glomerulus is larger in diameter than its efferent arteriole.

In compensatory hypertrophy, increase in glomerular and renal size is reputedly due to simple hypertrophy of glomeruli with hyperplasia and hypertrophy of the cells of the tubules and not to regeneration of new elements. Vascular constrictions were not apparent in any of the kidneys that did not undergo hypertrophy, and may be the result of a more tortuous course on the part of the vessels, due to their increase in caliber and length.

SUMMARY

1) Blood to the canine kidney enters the renal artery from the aorta, goes through end arteries, interlobar vessels, arcuate vessels, interlobular arteries, and finally to glomeruli via afferent arterioles. Efferent arterioles (arteriolae rectae spuriae) leave the glomeruli and course directly into the outer layer of the medulla, giving rise to long capillary nets which extend to the apical end of the pyramid; or efferent arterioles branch into intertubular capillary networks directly.

2) Venous drainage of the kidney stems from the stellate veins in the capsule, which are very numerous in the dog. These empty into interlobular, arcuate, interlobar, and finally the main trunk of the renal vein, which discharges into the caudal vena cava.

3) A few isolated arteriolae rectae verae were found, going from the arcuate arteries directly into the medullary region. Ludwig's arterioles supplying peritubular plexuses directly from afferent glomerular arterioles were observed in some specimens. Arteriovenous anastomoses were nonexistent. Afferent and efferent glomerular arterioles anastomosed in a few instances.

4) A rare arterial connecting artery was found in the latex cast of a hypertrophied kidney, running from an interlobar artery to a joining arcuate artery, giving off branches to four other interlobar arteries.

5) In renal hypertrophy, individual vascular elements were enlarged. Compensatory constrictions occurred along the paths of many enlarged vessels.

6) Attempts to verify the "Trueta Phenomenon," *i.e.*, the normal existence of two independent circulatory pathways in the kidney, were unsuccessful. Observations indicated that separate outer cortical and juxtamedullary circulations did not exist.

7) Measurements over a series of vascular renal elements conflicted with Trueta's theorem that juxtamedullary vasa efferentia are much larger than efferent vessels from outer cortical glomeruli.

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The Propagation of Lymphoid Tumors in the Anterior Chamber of the Chicken Eye

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MANY INVESTIGATORS have used the anterior chamber of the eye to study the characteristics of tumors. (For a review of earlier literature, see Lucké and Schlumberger.[15]) By means of a slit lamp microscope directed through the cornea, Lucké and Schlumberger[15] and Schlumberger and Lucké[19] studied the manner and rate of growth of tumors in the anterior chamber of the leopard frog (*Rana pipiens*). Greene and co-workers[8-12] investigated extensively the growth characteristics of various heterologous tumors in the anterior chamber of several species of animals. Results obtained by them supported the theory that transplantations of autonomous tumors of heterologous species always grew in the anterior chamber of the eye, whereas transplantations of nonautonomous tumors did not. In attempts to confirm these data, Dyer and Kelly[7] were able to obtain growth in heterologous species with only seven of 14 clinically proved cancers.

Shrigley *et al.*[20, 21] found that the Rous chicken sarcoma grew in the anterior chamber of the guinea pig eye and the mouse eye. The tumor virus survived the passages in the mammalian host, and its tissue specificities were apparently altered. Shrigley[22] was able to grow five other avian fibrosarcomas in the guinea pig eye.

The chicken eye has been used little thus far as a culture medium for studies of the growth of either homologous or heterologous tumors. Mori (as cited by Gyotoku[13]) obtained growth of the Fujinami's sarcoma in the chicken eye with cellular material but not with filtrates. Recently, in a preliminary report by Little, Woll, and Sampath,[14] it was stated that cellular suspensions of Rous sarcoma and of the avian lymphoid tumor strain RPL 21 introduced into the anterior chamber resulted in tumor growths. Concurrently, Burmester and Belding[6] reported the growth of two avian lymphoid tumor strains (RPL 12 and RPL 16) in the chicken eye.

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In avian lymphomatosis, one form of the disease (ocular lymphomatosis) is expressed by infiltration of the iris, ciliary body, and, to a lesser extent, other vascular parts of the eye with mononuclear cells similar to those found in other forms of this disease. Since it was found that tumors of naturally occurring visceral lymphomatosis can be propagated either with a suspension containing viable tumor cells or with filtered tumor extracts,[1, 4, 5] it was of interest to determine the type of tumor involvement or other alterations that resulted upon the injection of tumor cells and tumor extracts into the anterior chamber of the chicken eye. A complete report of these studies is presented here.

EXPERIMENTAL

Tumor tissue used in the preparation of inoculums for injection into the anterior chamber was obtained from two avian lymphoid tumor strains RPL 12 and RPL 16. Both strains originated from cases of naturally occurring visceral lymphomatosis. Strain RPL 12 tumor was artificially transferred from chicken to chicken with cellular suspensions at least 138 times at the Massachusetts Agricultural Experiment Station[4-6] before it was obtained by this laboratory. Here, it was transferred at least 150 times prior to its use in these experiments. This tumor contains a filtrable agent which produces a lymphomatous involvement of the viscera and osteopetrosis after a relatively long incubation period.[2, 3] Strain RPL 16 was developed at this laboratory[1] and had been propagated through fifty passages prior to its use in the present experiments. This tumor also contains a filtrable agent, though its pathogenicity appears to be variable.

Both tumor strains were chosen for these experiments because they were highly malignant. Cellular inoculum caused tumors to develop in all birds at the site of injection in five to ten days, and usually death followed soon with tumorous involvement of the viscera. Inoculations with filtrates resulted in tumors after an incubation period of sixty to 150 days; however, such tumors developed only in the viscera and not at the site of injection.

Liver, thymus, and bursa of Fabricius from

grossly normal chickens were pooled in approximately equal proportions by weight and used as a source of material for control inoculations.

The first inoculations into the eye were made with preparations of intramuscular tumor tissue and with normal lymphoid tissue which had been frozen and stored at —76 C. For all subsequent inoculations, tumors or other pathological alterations that had developed in the eye were used as the source of material for further inoculations. Inoculation tests for the presence and activity of a filtrable agent were made of frozen intramuscular tumor and of several intraocular tumors taken from birds used in the intraocular serial passage of the two lymphoid tumor strains.

Two types of inoculums were prepared and used for all eye inoculations, *i.e.*, crude tumor suspensions which contained a large number of viable tumor cells and tumor extracts which were rendered cell-free by several different procedures.

The tumor cell suspensions were prepared by first passing the tumor through an Olson[10] mincer or a TenBroeck grinder and then suspending it in ten parts by weight of 0.85 per cent NaCl solution. The inoculums for the first passage of both tumor strains were allowed to stand in a cylinder and after ten minutes the supernatant was decanted and used. For all other inoculations with cellular material, the original suspension was filtered through two layers of sterile cheesecloth prior to inoculation.

The cell-free tumor extracts were prepared by passing the tumor through an Olson mincer, freezing and thawing rapidly two times, and then processing in a TenBroeck grinder or a Potter's test tube homogenizer with one to two parts of 0.85 per cent NaCl solution. The extracts were further diluted to ten times the original weight of the tumor and then centrifuged for ten minutes at an R.C.F.* of about 20,000. The supernatants were used either directly or, as in the last passages of both strains, were first passed through a No. 8 Horman sterilizing filter pad.

Single Comb White Leghorn chickens, obtained from pedigree matings of three inbred lines selected for their susceptibility to naturally occurring lymphomatosis,[23, 24] were used in the inoculations to be reported. Chickens of different lines and matings were distributed among the various lots in any one experiment, so that any differences in genetic constitution were equalized as nearly as possible. The chickens were about 1 week old when inoculated.

Inoculations were made into the anterior chamber with a 27-gauge needle and a ¼-cc. tuberculin syringe. The point of entry was at the lateral edge of the cornea, 1-3 mm. from the corneal-scleral juncture. The needle was directed medially and about 0.02 ml. of inoculum was deposited in the anterior chamber Usually, some aqueous humor escaped upon the withdrawal of the needle.

The eyes were examined at weekly intervals. All birds that died or were killed at the termination of each experiment were subjected to autopsy. Representative pathological specimens were obtained from birds that were killed. The whole globe was placed in Zenker's formol fixative for about an hour, then small windows were cut into opposite sides of the posterior compartment so that there was free circulation of fixative in this part of the eye. Fixation was continued for approximately twenty hours, after which the eye was washed, dehydrated, and embedded in paraffin with the aid of reduced pressure. Sections were cut on a rotary microtome and stained with Ehrlich's alum hematoxylin and triosin.

Results and Discussion

The first inoculations which were made with frozen intramuscular tumors produced pathological alterations similar to those resulting from subsequent inoculations with unfrozen intraocular tumors. The results obtained with the two lymphoid tumor strains were also remarkably similar. This was true with respect not only to the pathological alterations but also to the incubation period and rate of occurrence. However, the two types of inoculums used, *i.e.*, tumor cell suspensions and cell-free preparations, produced distinctly different lesions. Alterations observed in the inoculated eye of chicken K716T are typical of results obtained with tumor cell suspensions. Photomicrographs of this eye are given in fig. 3 and 4.

Results with Tumor Cell Suspensions.— Grossly visible neoplastic changes occurred in the eyes of most birds inoculated with the crude tumor suspensions. Within eight days after inoculation, such birds had an enlargement of the eyeball, especially of the anterior portion, yellowish material usually appeared in the anterior chamber, and the cornea often showed some cloudiness. The lower eyelid became edematous and hyperemic and, in most cases, there was excessive lacrimation.

Microscopic studies revealed the presence of extensive tumor growth in the iris and ciliary body, with some extension along the choroid coat (*see* fig. 3). Innumerable single and grouped tumor cells were free

*R.C.F. = relative centrifugal force or force in terms of gravity = $.0000284RM^2$, where R = radius of centrifugation and M = revolutions per minute.

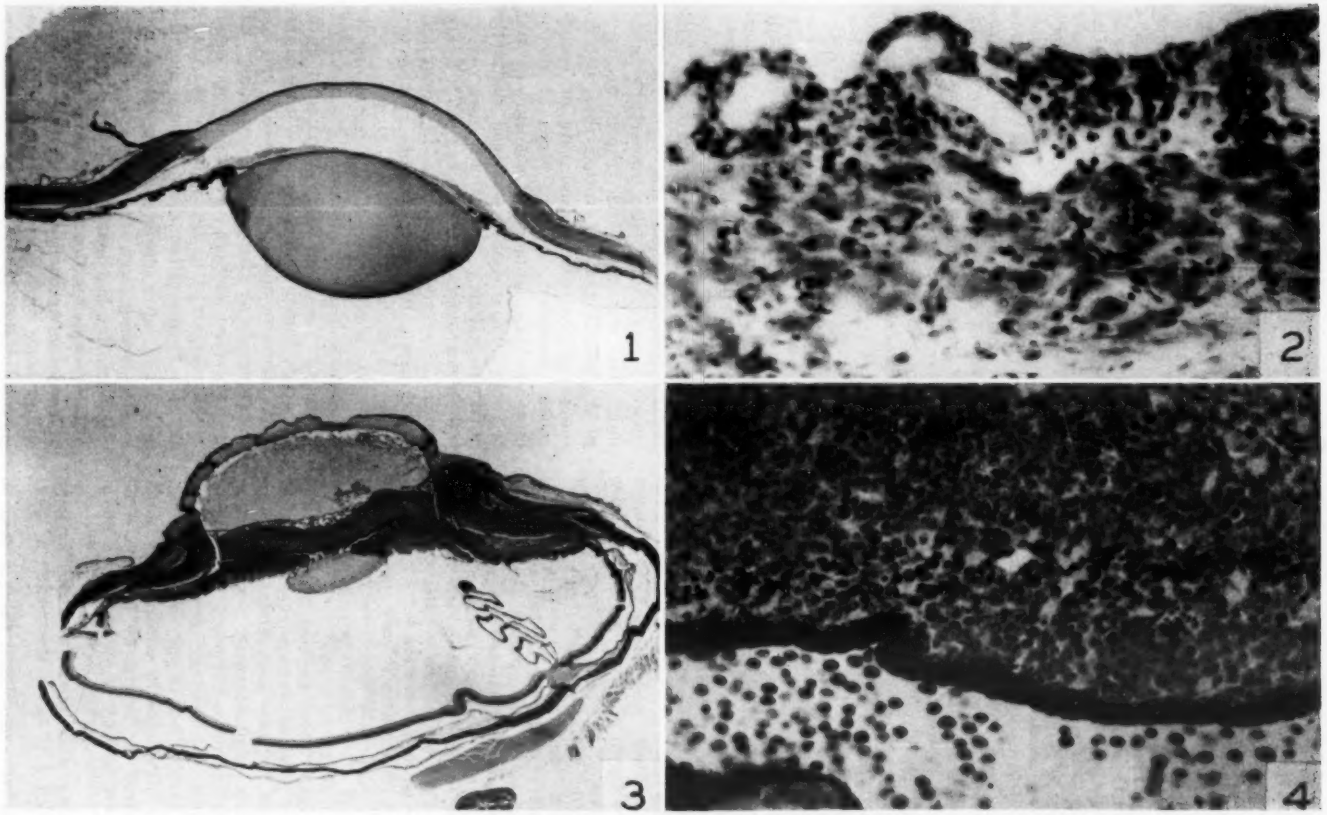

Fig. 1—A horizontal section of the anterior portion of an eye showing the typically abnormal gray condition of the iris. x 8.
Fig. 2—High power view of the iris of the eye shown in fig. 1. x 340.
Fig. 3—An off-center horizontal section of the eye that had been injected with a suspension of tumor cell. Note the extensive tumorous involvement of the iris and ciliary body. x 10.
Fig. 4—High power view of the iris in the eye shown in fig. 3. x 340.

in the aqueous humor of both the anterior and posterior chamber and some were adherent to the inner surface of the cornea. The size of the anterior chamber was greatly enlarged; the cornea bulged and showed eroded areas on the outer surface. The characteristics of the tumor cells were similar to those obtained by regular intramuscular passage.[1] Numerous mitotic figures are also evident in fig. 4.

The percentage of the inoculated birds which developed intraocular tumors and the average survival time are given in tables 1 and 2 for strains RPL 12 and 16, respectively. Results show that, with one exception, a high percentage (63 to 100%) of eyes injected with crude tumor suspension developed a grossly visible tumor which, in most cases, resulted in metastasis to the viscera and death. The one exception occurred in the first passage of strain RPL 12. The supernatant of the Olson minced tumor did not produce obvious tumors in any of 8 inoculated birds. The reason for this has not been determined; however, it may have been due to the fact that the tumor tissue was divided into such coarse particles in passing through the Olson mincer that practically all the tumor settled out in the ten-minute standing time. However, such was not the case with strain RPL 16, since inoculum prepared by the same procedure produced tumors in 85 per cent of 13 eyes inoculated. The average survival period among the various groups inoculated with crude tumor suspension varied from 10.6 to 14.9 days for strain RPL 12 and from 8.0 to 11.5 days for RPL 16.

Results with Cell-Free Inoculums.—An abnormal grayness of the iris developed in a variable percentage of birds injected with tumor filtrates or centrifuged supernatants. The abnormal irides were highly variable in form as well as in color. Some merely had an irregular pupillary border, some were constricted to a varying degree, and others were less mobile than normal, or entirely fixed. The amount of grayness, or rather the lack of yellow, varied from a slight to a complete lack of color and was either uniform throughout the iris or was irregular with isolated areas lacking the normal color.

Microscopic examination of 11 cases having typical gray irides revealed that the primary pathological alteration was a degeneration of the striated muscles of the irides. The extent of degeneration was variable and included such changes as hyalinization and granular or vacuolar degeneration of the striated muscle cells. In many cases, there was a number of small foci of lymphocytes in each section or more than the usual number of lymphocytes scattered in several areas of the iritic tissue (fig. 1 and 2). In no case was there a sufficient accumulation of these cells to warrant a diagnosis of ocular lymphomatosis.

The percentage of birds that developed grayness of the irides varied between different experiments from 11 to 83 per cent (tables 1 and 2). This condition was found only in chickens inoculated with cell-free preparations, except that in the first passage of strain RPL 12, 4 cases occurred in chickens injected with tumor cell suspensions, and 3 of 9 birds injected with minced normal lymphoid tissue also developed gray irides. The abnormal gray condition of the irides did not persist. It usually appeared in three to six weeks after inoculation, and in only a few cases did it remain visible three months after inoculation. Thus, it appears to be a transitory condition which

TABLE 1—Results of Inoculating Various Preparations of a Lymphoid Tumor (Strain RPL 12) into the Anterior Chamber of the Chicken Eye

Passage	Preparation of inoculum	No. inoculated	Ocular tumors		Gray iris (%)
			%	Average survival period (days)	
1	Normal tissue, minced	9	0	...	33
	Frozen pectoral tumor,				
	a) Olson, minced, supernatant	8	0	...	25
	b) TenBroeck, ground suspension	19	63	14.0	11
	c) TenBroeck, ground supernatant	19	0	...	16
2	Ocular tumor, suspension	13	93	14.9	0
3	Ocular tumor, suspension	12	92	10.6	0
4	Ocular tumor, suspension	5	100	13.0	0
5	Ocular tumor, suspension	9	100	11.3	0
	Ocular tumor, filtrate	18	0	...	83
6	Gray eye, filtrate	18	0	...	17
	Normal lymphoid tissue, filtrate	24	0	...	12

TABLE 2—Results of Inoculating Various Preparations of a Lymphoid Tumor (Strain RPL 16) into the Anterior Chamber of the Chicken Eye

Passage	Preparation of inoculum	No. inoculated	Ocular tumors		Gray iris (%)
			%	Average survival period (days)	
1	Frozen pectoral tumor,				
	a) Olson minced, supernatant	13	85	9.1	0
	b) TenBroeck, suspension	28	64	11.5	0
	c) Ground and frozen, supernatant	25	0	...	12
2	Ocular tumor, suspension	17	82	10.4	0
3	Ocular tumor, suspension	13	92	9.4	0
	Ocular tumor, supernatant	26	4	8.0	0
4	Ocular tumor, suspension	9	100	9.3	0
	Ocular tumor, filtrate	22	0	...	27

is in contrast to the usual case of ocular lymphomatosis.

The microscopic picture obtained could easily have been due to a nonspecific reaction of the tissue to the foreign material injected into the anterior chamber. The clinical data are in agreement with this possibility, inasmuch as the occurrence of the gray eye condition was quite variable between groups—it was transitory, and it appeared in groups injected with normal chicken tissue.

It is evident that tumor cell suspensions of the two lymphoid tumor strains RPL 12 and RPL 16, when injected into the anterior chamber of the chicken eye, induced the growth of tumors in the iris, ciliary body, and adnexa. However, filtered or centrifuged extracts of the same tumors did not induce typical ocular lymphomatosis during the experimental period of about a hundred days but produced a transitory graying of the iris in a variable percentage of birds.

SUMMARY

Cellular suspensions prepared from tumors of the avian lymphoid tumor strains RPL 12 and RPL 16, when injected into the anterior chamber of the chicken eye, resulted in tumors in the iris, ciliary body, and adnexa. These tumors were propagated through six (RPL 12) and four (RPL 16) passages in the chicken eye. Cell-free tumor extracts prepared from tumors used in the first and last passages of each tumor strain produced an abnormally gray iris which appeared three to six weeks after inoculation and disappeared in about eight weeks. This condition also appeared in eyes injected with preparations of normal lymphoid tissue and in eyes of two lots injected with cellular material. On the basis of the clinical results and histopathological alterations obtained, it was concluded that the abnormal graying of the iris was the result of nonspecific reaction to the foreign tissue injected and could not be considered as an early stage in the development of ocular lymphomatosis.

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Chronic Respiratory Disease of Chickens

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CHRONIC RESPIRATORY disease (CRD) of chickens was first described by Delaplane and Stuart[1] in 1943. The salient characteristics described for the disease were nasal discharge, respiratory râles, slow rate of spread, persistency of the symptoms, moderate decline in egg production, and loss in body weight. The etiological agent was observed to be a virus-like organism which was propagated in embryonating chicken eggs. Approximately 25 to 50 per cent of the embryos died after an incubation period of twenty-four hours, whereas the remainder, in most instances, survived until hatching time. Cutaneous hemorrhages and enlarged, congested, hemorrhagic kidneys were observed in embryos that died early. Whitish, circumscribed foci on the chorioallantoic membranes and renal congestion with deposition of urates were manifested by embryos surviving for several days. In some cases, the liver was mottled and enlarged or showed whitish foci.

Young chicks and mature birds inoculated with the egg-propagated agent or with the field virus manifested symptoms six to seventeen days after exposure, and the course of the disease varied from twenty-one to sixty-eight days. Only a coryza was observed in chicks either experimentally or naturally infected, whereas both coryza and respiratory râles were present in mature birds. Recovered birds were refractory to reinoculation with the agent.

Later studies by Delaplane[2-5] on isolated field strains revealed that lesions produced in embryos were not as consistent in character as hemorrhages of the amnionic and yolk sac membranes. Cutaneous, cranial, and renal hemorrhages were evident in some embryos. Diffuse radiating or localized opaque areas, with or without appreciable thickening, were seen in the chorioallantoic membranes. Fibrinous exudate was found occasionally around the abdominal organs. One strain of virus produced arthritis in the embryos, which was considered nonspecific for the agent. The virus was inactivated by streptomycin. On the basis of growth pattern in embryos and on the basis of symptoms and lesions produced in turkeys and chickens, the viruses of turkey sinusitis and CRD were considered indistinguishable.

Johnson[6] has reported a respiratory disease that occurred chiefly among broiler chicks 6 to 10 weeks of age. Slight nasal discharge was noted, but tracheal râles, with a more or less persistent hacking cough, were most frequently observed. Reduced feed consumption, retarded growth, persistency of the respiratory symptoms, and 10 to 20 per cent mortality were included in the syndrome of the disease. The lesions were confined largely to the air sacs and lungs. A marked thickening of the air sac membranes and massive accumulation of caseous pus in the air sac cavities were described. This disturbance was reported as unrelated to other common respiratory infections in chickens but was considered to be closely related, if not identical, to infectious sinusitis in turkeys.

The etiological agent was believed to be a rickettsial-like organism which could be propagated in embryonating eggs and turkeys, producing a sinusitis in the latter. Sinus exudate from affected turkeys, when inoculated into the air sacs of chickens, reproduced the disease. Streptomycin and aureomycin exerted an inhibitory effect on the agent.

Reagan, Brueckner, and Delaplane[7] reported the virus of CRD in chickens to have an average mean diameter of 53 mμ, as compared to 60 mμ for the virus of infectious sinusitis of turkeys.

In 1949, Delaplane identified the chronic respiratory disease agent (CRDA) in one flock in Massachusetts. During the past several years, respiratory disease outbreaks have occurred in numerous poultry flocks that have been immunized against Newcastle disease and infectious bronchitis. These respiratory disease infections have been identified infrequently as "breaks" in the vaccination programs or labelled as unknown respiratory infections. Since September, 1950, a concerted effort has been made to isolate and identify the causative respiratory disease agent in flocks with histories suggesting a CRD infection. In this paper are given the pertinent observations obtained from the flock histories, bird necropsies, and the results of embryo- and bird-inoculation studies.

PROCEDURE

During the period of this study, a total of 60 active respiratory cases was submitted

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to the laboratory for identification of the causative agent. The flock histories of the cases, which were identified as positive to CRD, were analyzed and the pertinent observations recorded. The gross pathological changes in 136 birds, representing 53 flocks affected with the CRDA, were summarized. A positive diagnosis was made on the basis of embryo- and bird-inoculation studies.

The general techniques for embryo- and bird-inoculation studies are described briefly. Tracheal scrapings from chickens suspected of having the disease were suspended in beef extract broth (exudate from one trachea per 5 ml. of broth) and treated with penicillin or penicillin and streptomycin for embryo inoculation. The dosages of antibiotics employed were 10,000 units of penicillin and 25 mg. of streptomycin per milliliter of suspension. Streptomycin was used mainly to inhibit some of the bacteria that were not inhibited by penicillin. Streptomycin, when used in the above concentration, did not completely inhibit the growth of the CRDA. However, the CRDA was most frequently isolated from tracheal suspensions that had been treated only with penicillin. Six- or 7-day-old embryos were used for inoculation, the dose being 0.2 ml. via the yolk sac route. Embryo mortality occurring within twenty-four hours after inoculation was not considered significant. The allantoamniotic (a.a.) fluids were harvested from embryos dying between the second and fourteenth days after inoculation, stored at —26 C., and used for further embryo- or bird-inoculation studies. The embryos which survived the full length of the natural 21-day incubation period for the chicken embryo were examined for gross lesions.

Two- to 3-month-old chickens immune to Newcastle disease and infectious bronchitis were selected for inoculation. In the majority of cases, one pooled suspension from each consignment was inoculated into 2 chickens. For bird inoculation, the tracheal exudate suspension was treated only with penicillin (10,000 units per ml. of suspension). In some cases, the material was inoculated only into turkeys and, in a few cases, into both turkeys and chickens. The trachea and a single sinus were used as the routes of inoculation for chickens and turkeys. In some cases, only a single route of inoculation was used, either the trachea or the sinus. The dose per route was 0.5 ml. of the penicillin-treated suspension or a.a. fluids containing the CRDA. The inoculated birds were observed for two to three weeks for clinical symptoms.

The influence of the various antibiotics on turkeys and chickens artificially infected with the CRDA was studied.

The effect of CRDA on mice was also determined.

Results

Study of the Natural Disease Outbreaks.—The disease was observed in flocks ranging from 2 to 18 months of age and was reported in birds 3 weeks of age. Predominating symptoms included respiratory râles, nasal discharge, loss in body weight, and a decline of 10 to 40 per cent in egg production. The severity and persistence of symptoms varied markedly. The resumption of normal egg production was very slow and, in some cases, did not return to normal. Birds affected prior to attaining egg production were delayed somewhat in their sexual maturity. The initial production rate was low for a prolonged period. Egg quality in affected birds was usually normal but, in some cases, a slight to moderate change in color and texture was observed. A 10 to 20 per cent decline in feed consumption in affected flocks was noted in some instances. Climatic conditions appeared to influence the severity of symptoms.

The disease was observed to occur frequently on premises on which flocks were affected in previous years. In some cases, the disease did not spread to the mature birds that had been on the premises for one or more years. The disease was noted in birds on the range and in the laying house, but was observed usually in the latter. Infrequently, the symptoms were reported as appearing after exposure of the birds to either Newcastle disease or bronchitis vaccines. In many cases, the infection spread slowly and appeared to be confined to certain pens of birds in the flock. One flockowner noted relatively high mortality in chicks that were hatched from affected birds. The mortality occurred especially up to 6 weeks of age. The influence of CRD on egg fertility and hatchability, to be properly evaluated, should be investigated further.

In several cases, where chickens and turkeys had been raised on the same premises, infectious sinusitis was diagnosed in the turkeys and CRD was identified in the chickens.

Disease Syndrome in Naturally Infected Birds.—The gross pathological changes in 136 birds representing 53 flocks affected with CRD are summarized in table 1. The incidence of symptoms and lesions among these birds has been listed according to the age of the bird. Active respiratory symp-

TABLE 1—Incidence of Pathological Changes in Birds of Various Ages Obtained from 53 Flocks Naturally Infected with the Chronic Respiratory Disease Agent

Pathology	No. flocks	No. birds	Age of birds (months)										
			2	3	4	5	6	7	8	9	10	11	12-18
Tracheal râles	53	121	27	21	6	4	11	5	8	5	8	4	22
Nasal discharge	14	18	0	2	1	0	3	0	2	1	1	3	5
Rhinitis	29	55	6	10	4	0	6	3	4	2	8	1	11
Sinusitis	28	45	3	9	5	0	3	2	2	3	5	3	10
Laryngitis	48	111	19	20	7	3	12	5	6	4	9	3	23
Tracheitis	53	132	27	23	8	4	12	5	10	5	10	4	24
Bronchitis	20	28	2	6	1	1	1	3	1	2	6	0	5
Pneumonia	15	18	2	6	1	1	1	0	0	2	2	0	3
Aërosacculitis	35	57	8	13	2	4	2	2	3	3	6	2	12

toms were noted in 90 per cent of the birds. The incidence of lesions in birds submitted for laboratory examination was observed as follows: rhinitis, 40 per cent; sinusitis, 33 per cent; laryngitis, 82 per cent; tracheitis, 97 per cent; bronchitis, 21 per cent; pneumonia, 13 per cent; and aërosacculitis, 41 per cent. Approximately, 50 per cent of the birds submitted for laboratory examination were immune to Newcastle disease. The ages of the chickens ranged from 2 to 18 months and apparently had no influence on the incidence of the manifestations of the disease. The character and degree of the pathology was nonspecific and indistinguishable from infectious bronchitis and Newcastle disease, except in some cases where the tracheal exudate was very turbid and cohesive. Pneumonia was seldom extensive and was confined usually to the posterior dorsolateral border of the lungs. Aërosacculitis was manifested usually by a thickening and opacity of the air sac membranes, which might be coated with yellowish, creamy exudate. Considering the types of symptoms and the character of lesions produced, one is not likely to make an accurate differential diagnosis on ante- and postmortem examination of chickens affected with CRD. An accurate diagnosis can be approached by obtaining an adequate flock history, isolating the CRDA in embryos, and reproducing the disease in chickens.

Propagation of the CRDA in Embryos.—The mortality pattern and embryo lesions were observed to be very similar to those reported by other investigators. Seven-day-old embryos were found most satisfactory for propagation of the CRDA since, in several cases where 10-day-old embryos were used, the mortality rate was found to be low. It should be noted that on occasion some difficulty was encountered in propagating the agent in the 7-day-old embryo. When the inoculated embryos were permitted to incubate the full length of the natural incubation period, significant mortality and respiratory lesions sometimes developed during the terminal period of incubation. Caseous exudate was observed in the trachea, bronchi, and lungs. Air sac membranes were thickened, opaque, and coated with caseous exudate. The respiratory lesions were produced by 26 of the 53 CRDA isolates. This figure might not be entirely accurate because, at the onset of these studies, the embryos were removed several or more days prior to hatching. These lesions, frequently evident on first passage and also following ten serial embryo passages of the agent, were observed only in embryos that had been inoculated with the CRDA or the turkey sinusitis agent.

Another lesion evident only in embryos infected with the CRDA was an abscess-like process which developed in the region of the articulations of the appendages and, infrequently, of the mandibles. This lesion sometimes occurred in combination with an opacity of the cornea. The abscess-like process was produced by 16 of the 53 CRDA isolates and was observed to appear usually after two or more embryo passages. This lesion was observed only in embryos that had been inoculated with the CRDA.

The lethal titers for embryos of several CRDA isolates were determined. Undiluted and tenfold dilutions of the a.a. fluids were used. Embryo mortality that occurred within twenty-four hours postinoculation was regarded as insignificant. The criterion for calculating the titer of the CRDA was 50 per cent mortality of the embryos during the fourteen days following inoculation. Several of the isolates were found to titer at least 10^{-6} in chicken embryos.

A comparative study was made on the behavior of the agent in embryos. Thirteen isolates that had been propagated in embryos for two or more passages were uti-

TABLE 2—Lesions in Embryos That Died Following Inoculation at 7 Days of Age with Thirteen Different Isolates of the Chronic Respiratory Disease Agent

	Incidence of lesions (days postinoculation)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total
Mortality*	22	12	15	11	20	27	28	21	29	22	22	37	36	32	23	357
No. embryos examined	0	3	2	6	13	18	28	17	25	21	22	36	36	32	23	282
Stunting	..	1	..	4	4	4	9	9	17	17	9	5	8	4	3	94
Edema	..	..	1	6	9	12	20	7	3	1	..	..	..	..	..	59
Congestion	..	2	1	6	10	13	23	11	6	1	..	..	..	..	..	73
Hemorrhage	..	..	..	1	7	8	8	3	1	..	..	..	..	..	..	28
Abscesses**	..	..	..	..	..	2	8	4	3	2	2	1	..	..	..	22
Liver lesions***	..	..	..	3	3	11	16	12	21	19	15	21	24	18	18	181
Kidney lesions	..	..	..	1	2	8	10	13	20	10	18	15	14	9	14	134

*A total of 485 embryos were inoculated; of these 357 died, but only 282 were examined. Seventy-five embryos were not examined because of early mortality and other reasons. **Abscess-like process occurring in the region of the articulations of the appendages. ***Liver and kidney lesions included congestion, necrosis, and enlargement.

lized in this study. The a.a. fluids were inoculated undiluted or in dilutions of 10^{-1} and 10^{-2}. Mortality was delayed or did not occur in the higher dilutions. Lesions were observed in embryos inoculated with the various dilutions of a.a. fluids. Stunting, hepatic, and renal changes were noted four to fifteen days postinoculation. Cutaneous and subcutaneous lesions, that is, edema, congestion, and hemorrhage, were observed two to ten days postinoculation. The abscess-like lesions were observed six to twelve days postinoculation. Respiratory lesions were noted as early as nine days postinoculation, with the highest incidence observed in embryos examined thirteen to fifteen days postinoculation. These respiratory lesions were most frequent in pipped embryos or chicks. The distribution and incidence of these lesions are listed in tables 2 and 3.

Infrequently, lesions were observed in the chorioallantois and in the yolk sac during the mid and terminal postinoculation period. The chorioallantoic membranes were edematous and irregularly opaque and the yolk sac was congested and hemorrhagic.

Bird-Inoculation Results.—Table 4 is a summary of the gross pathological changes observed in 81 birds after inoculation of the CRDA into the trachea and sinus (0.5 ml. each). The birds were killed at the end of the clinical observation period (14 to 21 days postinoculation). Thirty-five different suspensions containing the CRDA were used. The remaining CRDA isolates were inoculated into birds by different routes and, for this reason, the results have not been included in the table. Approximately, 50 per cent of the birds inoculated with the treated (penicillin) tracheal exudate suspension or a.a. fluids containing the CRDA showed respiratory symptoms. Symptoms appeared in these birds between four and eighteen days postinoculation and continued throughout the observation period. The course of the disease was not determined. Tracheal râles were noted most frequently among the symptoms recorded. Respiratory lesions of various extent and degree were observed in all but 2 of the inoculated birds.

The reaction produced in the sinus might be described as a general edema of the sinusoidal mucosa plus an excess accumulation of slightly turbid, viscid exudate (fig. 1). The mucosa often exhibited a yellowish cast. The tracheal mucosa might be thickened, edematous, and coated with viscid, turbid exudate. The upper trachea might be congested slightly in some instances, and infrequently the mucosa might appear roughened. The shoulder joints in-

TABLE 3—Incidence of Respiratory Lesions in Embryos and Chicks Following Inoculation with Thirteen Different Isolates of the Chronic Respiratory Disease Agent

Embryo condition	No. embryos examined	Incidence of lesions* (days postinoculation)						
		9	10	11	12	13	14	15
Dead—nonpipped	232	3	1	5	3	12	5	4
Dead—pipped	50	..	..	..	..	2	22	17
Alive—nonpipped	21	..	..	1	..	..	2	..
Alive—pipped	35	..	..	..	..	..	14	11
Hatched—chicks	72	..	..	..	..	..	19	4

*Respiratory lesions may include tracheitis, thickened air sac membranes, or lung involvement.
Note: A total of 485 embryos was inoculated; of these 357 died, but only 282 were examined. The 56 live embryos and 72 chicks which hatched were also examined for respiratory lesions.

TABLE 4—Correlation of Symptoms and Lesions in 81 Birds Inoculated with Thirty-Five Isolates of the Chronic Respiratory Disease Agent

Birds showing symptoms		Lesions listed by No. of birds showing the specific lesion or combination of lesions											
Symptoms	No. birds	S	T	A	S and T	S and A	S T and A	A and T	T P and A	S T P and A	S P and A	S T and P	No lesions
No symptoms	33	4	1	3	4	1	12	1	0	5	1	0	1
Sinusitis	7	0	0	0	1	1	3	0	0	1	0	0	1
Tracheal râles	36	0	2	0	3	1	16	1	2	9	1	1	0
Râles and sinusitis	4	0	0	0	0	0	2	0	0	2	0	0	0
Nasal discharge	1	0	1	0	0	0	0	0	0	0	0	0	0
Totals	81	4	4	3	8	3	33	2	2	17	2	1	2

S = sinusitis; T = tracheitis; A = aërosacculitis; and P = pneumonia.
Note: The birds were killed between fourteen and twenty-one days following inoculation.

frequently contained a frothy, turbid exudate. The air sacs, particularly the posterior thoracic, lesser and greater abdominals might be involved extensively. The air sac membranes were thickened and coated with a yellow, viscid exudate or a yellow, cheesy exudate. The turkey sinusitis agent reacted similarly in laboratory-inoculated birds (fig. 2).

The CRDA, when inoculated into turkeys 4 to 8 weeks of age, produced definite respiratory râles within two to three weeks, accompanied on occasion by a distention of the inoculated sinus. On necropsy, these birds might reveal an edema of the sinusoidal mucosa with slight to extensive lower respiratory involvement. If these birds were held for a prolonged period, they developed a typical sinusitis (fig. 3). The most common clinical manifestation in mature turkeys artificially exposed to CRDA was a marked distention of the sinus accompanied infrequently by tracheal râles. On necropsy, these mature birds revealed an extensive involvement of the sinusoidal mucosa and of the upper and lower respiratory tracts. The turkey sinusitis agent behaved similarly in turkeys.

The results obtained in birds were in agreement, for the most part, with those obtained in embryos. In some cases, when the embryo test proved unsatisfactory, the diagnosis was based solely on the bird test.

In laboratory-inoculated birds, it was found that mature birds appeared to be more susceptible to the CRDA than young ones. The mature birds developed symptoms sooner than young birds. However, the air sac lesions in young birds were more extensive than in mature ones.

Response to Antibiotics.—Streptomycin, aureomycin, chloromycetin, and terramycin exerted an inhibitory effect on the CRDA in birds artificially inoculated with the agent.

Turkeys that had been artificially inoculated with the CRDA were treated with aureomycin and with streptomycin. The time of treatment was initiated four to sixty-four days following the observance of active clinical symptoms. It was found that the oral aureomycin, administered on the basis of 25 mg. per pound of body weight for seven successive days, had no beneficial effect in relieving the clinical sinusitis. The parenteral aureomycin (25 to 50 mg. per sinus) was effective, in some instances. However, it also produced a somewhat toxic effect for several days following inoculation. Streptomycin (50 to 150 mg. per sinus) was the most effective in treating the local sinus infection.

The therapeutic effect of the aureomycin, chloromycetin, and terramycin on chickens artificially infected with the CRDA was studied. Aureomycin (parenteral, 25 to 50 mg. per bird per day) had little or no therapeutic value when administered intramuscularly and intravenously. Treatment was initiated at the time of inoculation and continued for six successive days. The birds were held for fourteen days after inoculation with CRDA. Chloromycetin (150 mg. per bird per day) and terramycin (100 to 200 mg. per bird per day), when administered orally for the same time interval, had only slight therapeutic value. The birds in these studies ranged in age from 8 weeks to maturity.

The results were somewhat different when the treatment was started on the seventh day following inoculation. Chloro-

Fig. 1—Mature hen inoculated by the intrasinus and intratracheal routes with allantoamniotic fluids containing the chronic respiratory disease agent. Distention of the right sinus, nasal discharge, and tracheal rales were evident seven days postinoculation when the photograph was taken.

mycetin (150 to 200 mg. per bird per day) was of moderate therapeutic value when administered orally for seven successive days, starting on the seventh day following exposure. These birds were killed on the fourteenth day following inoculation with the CRDA. Oral administration of terramycin (200 mg. per bird per day) was more effective than the chloromycetin in its therapeutic value. There was a marked remission in symptoms by the fourteenth day postinoculation. There was also a marked difference in the extent of respiratory lesions as compared to the lesions of the untreated birds. Mature birds and birds 8 to 11 weeks of age were used in these studies.

Studies in Mice.—One hundred and thirty-five mice (White Swiss and Leaden Hybrids) were inoculated with embryo-propagated strains of the CRDA. The age of the mice varied from 25 to 90 days. The routes of inoculation were intracranial, intraperitoneal, intranasal, intramuscular, and subcutaneous. No definite symptoms were observed and suspicious pneumonic lesions were noted only in several mice.

Discussion

Our knowledge of the prevalence and incidence of CRD is very limited. The disease has been observed in several widely scattered areas in this country. Johnson[6] reported that the disease causes marked retardation in growth in broiler flocks and that parts or all of the carcasses of birds slaughtered from affected flocks may be condemned because of pathological conditions caused by the disease. Others[5] have reported also that a significant number of broiler carcasses were condemned because of the extensive air sac pathology, which some have attributed to Newcastle disease infection either through natural outbreaks or through live virus vaccination. Since CRD may produce respiratory symptoms and lesions similar to those in other respiratory infections, it is essential that these respiratory pathological conditions in slaughtered chickens be subjected to the isolation of the etiological agent before a specific disease or diseases are incriminated. The economic importance of CRD should not be minimized.

The disease also may be of great economic importance when it appears in flocks that are raised or maintained for egg production. Our observations reveal that CRD may vary greatly in its behavior and severity. Affected flocks may recover from the respiratory symptoms but, in many cases, the disease follows a protracted course, which leads to unprofitable production. In the latter case, the flockowner often has difficulty in determining whether

Fig. 2—Mature hen inoculated by the intrasinus and intratracheal routes with allantoamniotic fluids containing the turkey sinusitis agent. A marked distention of the right sinus, nasal discharge, and tracheal rales were evident seven days postinoculation when the photograph was taken.

or not the flock should be liquidated, hoping that the disease may subside and that the birds may return to normal production. This moderate diminution in egg production, which leads to an unprofitable flock, should be recognized in appraising the economic importance of the disease.

Embryo and bacteriological studies have revealed that the disease is caused by a viral-like agent. It has been propagated with some difficulty in 7-day-old embryos and has produced a variable mortality among inoculated embryos, even after a number of successive passages. Its behavior in embryonating eggs has discouraged workers from adopting the embryo technique in the study and diagnosis of the disease. The agent does not produce gross pathological changes of striking significance in inoculated embryonating eggs, except in some instances when the embryos may manifest abscess-like lesions in the region of the articulations of the appendages and, infrequently, of the mandibles. Furthermore, caseous plugs in the trachea, bronchi, and air passages of the lungs have been observed frequently in inoculated embryos that are permitted to develop until the twenty-first day of incubation. Also, extensive and marked air sac lesions have been observed in embryos near the termination of the incubation period. These lesions appear to be of pathognostic value, although Hitchner[9] has described similar respiratory lesions in embryos inoculated with the turkey sinusitis agent. Our studies with the turkey sinusitis agent and CRDA revealed that both agents behave similarly in embryonating eggs. However, in respect to the behavior of other avian respiratory disease viruses in embryonating eggs, the respiratory lesions produced by the CRD and turkey sinusitis agents are of considerable diagnostic value.

Fig. 3—Ten-week-old poult inoculated into the right sinus with allantoamniotic fluids containing the chronic respiratory disease agent. A marked distention of both sinuses, nasal discharge, and coughing were evident twenty-eight days postinoculation when this photograph was taken.

Chickens inoculated with the CRDA usually develop a partial or complete syndrome of the disease. Birds, 2 to 3 months in age, when inoculated with the agent, usually develop an aërosacculitis which may be very pronounced. These pathological changes may be observed as early as seven days postinoculation and are well established after fourteen days. However, it was observed that in some cases the agent might not induce lesions by the fourteenth day postinoculation.

The results of the embryo tests and the bird inoculations agree very closely in the identification of the disease. Either procedure, used alone, may prove very serviceable in a diagnostic laboratory in the identification of the disease. One recognizes that a more simplified and economical test is desired, and it is hoped that a serological testing method may be developed for diagnostic and investigational purposes.

The CRDA is inhibited to some degree by streptomycin, aureomycin, chloromycetin, and terramycin. However, the degree of inhibition is not adequate for effective and economical therapeutic use in disease outbreaks.

The etiological agents for CRD and infectious sinusitis in turkeys have many characteristics in common, including the tinctorial properties of elementary bodies, which are demonstrable in sinus exudates and embryo material containing the respective agents.

SUMMARY

During the period of this study, the chronic respiratory disease agent (CRDA) was isolated 53 times from chicken flocks. Several of the distinguishing characteristics produced by this agent in chicken

embryos were: irregular mortality pattern, arthritis-like lesions, and respiratory lesions. Renal and hepatic lesions were observed frequently in embryos that had been inoculated with the CRDA.

The disease was reproduced by inoculation of Newcastle disease- and infectious bronchitis-immune birds with allantoamniotic (a.a.) fluids containing the CRDA or with tracheal exudate obtained from infected birds. In young birds (2 to 4 months) and in mature birds, the symptoms were sinusitis, rhinitis, and respiratory râles. In the mature birds, the symptoms, for the most part, were more marked than those in the young birds. Extensive and marked aërosacculitis was observed in young birds.

Streptomycin, aureomycin, chloromycetin, and terramycin exerted an inhibitory effect on the CRDA. Streptomycin was found the most effective for treating the local sinusitis reaction in turkeys, whereas terramycin (oral) was most effective in treating chickens inoculated with the CRDA. However, no practical treatment for chickens affected with the CRDA has been discovered thus far. Liquidation of the affected flock appears to be the only means of controlling the disease at the present time.

APPENDIX

Since this paper was submitted for publication, tracheal, pneumonic, and air sac lesions typical of chronic respiratory disease have been observed in embryos and chicks obtained from commercial flocks affected with the disease. The lesions were observed in embryos dead in the shell, among dead and live embryos which had pipped the shell, and in cull chicks 4 days or less in age. The CRDA was identified in these embryos and chicks by embryo- and bird-inoculation tests. These findings appear significant from the standpoint of dissemination of the disease via hatching eggs used for flock replacements or for the production of live vaccines for poultry.

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Avian Encephalomyelitis—Prevalence, Pathogenicity of the Virus, and Breed Susceptibility

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AVIAN ENCEPHALOMYELITIS (AE), first observed in 1930, has since become of considerable economic importance to the poultry industry, especially the broiler industry. This report deals with observations on (1) the incidence of the disease in the field, (2) the properties of the virus, and (3) its immunological aspects. For a review of the literature, the reader is referred to a paper by Jungherr and Minard.[7]

INCIDENCE

United States. — Avian encephalomyelitis can no longer be thought of as a disease localized in the New England area and a few additional states, as was the case a decade ago.[7] From a recent survey of states in which AE was previously unreported, it was apparent that the disease had spread generally throughout the country and had increased in economic importance, especially in the broiler areas.[1,2,3,5,14] Cox[2] noted that AE represented the following percentages of total diseases diagnosed in broilers in North Carolina for 1948, 1949, and 1950: 8.5, 2.4, and 4.9; and was third, eighth, and sixth, respectively, in relation to incidence. In Connecticut in 1950, AE accounted for over 3 per cent of all diseases diagnosed.

Connecticut.—Data for the period 1941 through 1950 showed the total number of cases diagnosed as 43, 26, 12, 29, 42, 13, 38, 108, 58, and 65 for the respective years. In order of decreasing importance, the following breeds and crosses were involved: sex-linked cross (SLX); broiler cross (BRX); Rhode Island Red (RIR); Barred Rock (BR); New Hampshire (NH); and White Rock (WR). With regard to hatcheries involved in cases diagnosed from 1941 through 1950 at the Storrs Diagnostic Laboratory, one hatchery was involved for all ten years; four for eight years; one for six years; and two for five years. For the above ten-year period, the extremes in average monthly mortality ranged from 0 to 41.6 per cent at the time of diagnosis.

For the years 1949 and 1950, the following data were noted: the earliest age at which symptoms appeared was 1 day of age; the maximum age was 42 days; the average for the first appearance of symptoms was 11.6 days.

Jungherr and Minard[7] noted a seasonal prevalence of the disease suggested by hatchery data. Confirmation that AE continues to show this epiornitic periodicity was evidenced by the ten-year data. Figure 1 shows the monthly incidence of AE in Connecticut for three typical years, which demonstrates the minor period of prevalence in the fall and the major one in the spring months.

PROPERTIES OF THE VIRUS

Preparation.—The original AE virus representing the 135th passage of this particular strain was obtained from Dr. H. Van Roekel.* Subsequent passages from this material were used throughout this work. A new technique of virus preparation from brain material[9] was evolved from one described by Kahnke,[8] because the mortar-grinding technique used previously[6] resulted in low-titer virus suspensions. Chick brains and buffered (M/15 phosphate) physiological saline pH 7.2 were added to 1- to 8-oz. medicine bottles (depending on the size of the desired brain pool), one fifth of the volume being composed of 3-mm. flint glass beads. Standard brain-saline dilutions were made up 1 : 10 by weight. The bottles were fastened horizontally in a Kahn** shaker and shaken for two hours at approximately 5 C., this length of time being necessary to rupture the brain cells. The suspensions were stored

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in 5-ml. aliquots at —20 C. Before inoculation, the suspensions were centrifuged at 1,500 revolutions per minute (r.p.m.) for five minutes and the supernatant used. Pools of brains were used wherever possible to give more uniform results. Routine passages of AE virus were made using the intracerebral (i.c.) route of injection. Each normal and AE brain pool was checked for bacterial and viral contamination by (1) streaking the material on suitable mediums and (2) injecting 6, 9-day embryonating eggs (0.2 ml. of inoculum per egg) by way of the allantoic chamber to determine the absence of Newcastle disease virus and infectious bronchitis virus.

A single strain of RIR chicks† was used in most of the experiments. Chicks were vaccinated at 1 day of age with commercial intranasal Newcastle disease vaccine in the right nostril and one drop of chicken-propagated infectious bronchitis virus, suspended in skimmilk to give a 1 : 20 dilution, in the left nostril. The chicks were held for a one-week convalescence period before injection. Control chicks, in parallel with experimental chicks, were injected with a normal brain suspension. At least 10 per cent of the clinically positive birds in each experiment were examined histologically for AE lesions. Calculating the 50 per cent end point,[15] the infective titer of the Van Roekel strain virus in our laboratory averaged $10^{-5.4}$.

Longevity.—Since our virus pools were stored at —20 C. and no reference was available as to longevity at this temperature, a large virus pool was prepared and periodically checked for virus livability by i.c. chick injection. Chicks were injected with 0.1 ml. of a 10^{-1} dilution of virus. Results indicated that the virus was still capable of infecting chicks after storage for 428 days.

Susceptibility to Antibiotics.—The effect of antibiotics was deemed important not especially from the therapeutic point of view, but more from that of eliminating bacterial contaminants in virus isolation and other laboratory work with AE virus. Penicillin and streptomycin at levels of 100,000, 1,000, and 100 units or μg. per milliliter and aureomycin at levels of 25, 2.5, and 0.25 μg. per milliliter, when added to and stored with an AE brain suspension (10^{-1} dilution), exhibited no inhibitory effect on AE virus when injected (0.1 ml.) i.c. into chicks. The antibiotics plus normal brains as controls proved negative.

Transmissibility to Embryonating Chicken Eggs.—Experiments were initiated in an attempt to cultivate the virus in embryonating chicken eggs so as to provide a more simple procedure than i.c. chick passages. Previous workers[7, 10, 17] had been able to hatch chicks manifesting the syndrome of the disease from eggs injected by various routes, but had been unable to show multiplication of the agent. Negative results were obtained in our work in attempts to show: (1) lesions on the chorioallantoic membrane after the inoculation of $10^{4.4}$ minimal infective doses (m.i.d.) of AE virus; (2) pathogenicity for embryos or chicks hatched after intra-amniotic injection with $10^{4.4}$ m.i.d. of AE virus.

In the first experiments using the yolk sac (y.s.) route of inoculating 6-day embryonating eggs (0.25 ml. of a 10^{-1} dilution of virus), a pleuropneumonia-like organism[16] was recovered which produced a high embryo mortality on repassage. Since this organism could not be isolated from the original AE virus inoculum, it was suspected that the organism was present in the eggs before inoculation. Later work using the y.s. route demonstrated that the virus could be recovered six and seven days after it had been injected, but was evidently not multiplying.

Duplicate zig-zag chick to embryo passages of AE virus, in attempting to adapt the virus for multiplication in chicken embryos, proved negative after 11 such passages. Injection was as follows: chicks—i.c. with 0.05 or 0.1 ml. of undiluted allantoic fluid; embryos—into the allantoic chamber with 0.2 ml. of a 10^{-1} virus dilution. In the course of these experiments, the viable virus was definitely shown to be present in the allantois four days postinoculation, contrary to the reports of previous workers.[7, 10] Presence of the virus was shown in embryos dead four, five, six, and seven days postinoculation and living embryos six and seven days postinoculation by injecting groups of 20 chicks i.c. with 0.1 ml. of undiluted pooled samples of allantoic fluid. Between 33.3 and 95.0 per cent of the injected chicks were clinically positive and histologically confirmed as having AE. The above data should add to those substantiating an egg-borne cycle of infection.[13]

A death pattern could not be produced by five or six "blind" serial passages in the allantois of 9- to 10-day-embryonating eggs. The presence of viable virus could be shown only in the first passage; the determination was made by i.c. injection of chicks with the allantoic fluid.

Transmission to Young Chickens Intraocularly.—Olitsky[11, 12] stated that in a few trials, injecting AE virus intraocularly (i.o.) proved a generally ineffective route of injection, although about 10,000 minimal chicken cerebral-infective doses were employed. In this study, the i.o. route was shown to be an effective method of transmitting AE to susceptible chicks, although not as effective as the i.c. route. Twenty 13-day-old RIR chicks were injected i.o. with 0.05 ml. of an AE brain suspension containing $10^{4.4}$ m.i.d., and 10

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control chicks received a like amount of a normal brain suspension. Over a period of two weeks, 18 of the 20 injected chicks appeared positive, showing bilateral ataxia. In the next experiment, 30 RIR chicks, 23 days old, were injected i.o. as described previously. Chicks were destroyed daily beginning three days postinoculation, and sections of right eye and lids, brain, proventriculus, pancreas, and spleen were fixed in Bouin's fluid for histological study.

In the first experiment, only 1 chick showed gross changes in the eye—a rectangular silver-colored patch (probably due to involvement of the lens)—while in the second experiment, 8 out of 30 showed this gross lesion. On histological study, 16 of 30 showed definite to marked infiltration by mononuclear cells (iridocyclitis) of the iris and frequently the ciliary body, although nine of these eyes appeared grossly normal. Brain lesions were typical of AE in chicks while controls were negative. The appearance of ataxia in i.o.-injected chicks occurred not more than one to two days after the expected appearance in chicks injected i.c. Table 1 shows the results of the complete study of the 30-chick experiment.

Transmissibility to Adult Chickens.—No report in the literature indicated the successful experimental infection of adult birds with AE virus. By using the intraperitoneal (i.p.) route of injection, infection of adult RIR birds, from the same strain as those used as chicks in the previous experiments and from 195 to 444 days of age (only range tested), was successfully accomplished. Progressive bilateral ataxia was the only constant clinical symptom noted. Amount of inoculum varied from 0.1 to 2.0 ml. containing $10^{4.4}$ to 20 x $10^{4.4}$ m.i.d., respectively. Table 2 shows the results obtained on 10 experimentally injected adult

TABLE I—Intraocular Injection with Avian Encephalomyelitis Virus

Bird No.	Examined	Gross pathology of the eye	Microscopic pathology											
			Eye	Eye lid	Optic nerve	Cerebellar molecular layer	Cerebellar nuc lei	Cerebellar white matter	Brain stem	Forebrain	Choroid plexi	Proventriculus	Pancreas	Spleen
1	K5	–	LF	–	–	–	–	–	–	–	–	–	–	–
2	K7	–	–	–	–	–	–	–	–	–	–	–	–	–
									VC					
3	A13	–	I	–	–	G	–	–	G	–	–	–	CF	–
4	K6	–	I	LF	–	–	–	–	–	–	–	–	–	–
							VC		VC					
5	A13	S	–	–	–	G	G	–	G	–	–	–	CF	–
6	K9	–	–	–	–	–	G	–	G	–	–	–	–	–
7	K8	–	I	LF	–	–	G	–	–	–	LF	–	CF	–
									VC					
8	K9	–	–	–	–	–	VC	–	G	–	–	–	–	–
									VC					
9	A12	S	I	–	–	G	G	–	G	–	–	–	–	–
10	K3	S	–	–	–	–	–	–	–	–	–	–	–	–
11	K5	S	I	–	–	–	–	–	–	–	–	–	–	–
							VC							
12	K7	–	–	–	–	G	G	–	G	–	–	–	–	–
							VC		VC	VC				
13	A14	–	I	–	–	G	G	–	G	G	–	–	–	–
14	A13	–	–	–	–	G	–	–	–	–	–	–	–	–
15	K6	–	–	–	–	–	–	–	–	–	–	–	CF	–
									VC	VC				
16	A14	–	I	–	–	G	G	–	G	G	–	–	CF	–
									VC					
17	A11	–	I	–	–	G	G	–	G	–	–	–	–	–
							VC		VC					
18	A14	–	I	–	G	G	G	–	G	–	–	–	–	–
19	A13	S	I	–	–	G	–	VC	G	VC	–	–	–	–
20	K7	S	I	–	–	–	–	–	–	–	–	–	CF	–
21	A13	–	–	–	–	G	G	–	VC	VC	–	–	–	–
							VC		VC					
22	A12	–	I	–	–	G	G	–	G	–	–	–	CF	–
23	K8	S	–	–	G	–	–	–	G	VC	–	–	CF	–
									VC					
24	K8	S	I	–	–	–	VC	–	G	–	–	–	CF	–
25	K9	S	I	–	–	–	G	–	G	–	–	–	–	–
26	A12	–	–	–	–	G	–	–	G	–	–	–	CF	–
27	K6	S	–	–	–	–	–	–	–	–	–	–	–	–
28	A13	–	–	–	–	G	G	–	G	–	–	–	–	–
							VC		VC	VC				
29	A14	S	I	–	–	G	G	–	G	G	–	–	CF	–
							VC							
30	K5	–	I	–	–	–	G	–	–	–	–	–	–	–

K = killed, number indicates days postinoculation; A = ataxia (clinically positive to AE), number indicates days postinoculation; sacrificed; – = normal; S = right eye pupil abnormal; CF = circumscribed follicles; G = gliosis; VC = vascular cuffs; I = iridocyclitis (infiltration by mononuclears); LF = lymphoid follicles.

birds. Two RIR adults, 444 days old, were injected i.p. with 0.8 and 1.0 ml., respectively, of a 10^{-1} dilution of AE virus. The first bird developed bilateral ataxia seventeen days postinoculation, the second, twenty-four days postinoculation, and each was killed at that time. One half of each brain was removed aseptically for processing by the "bead" technique, and the other half was fixed for histological examination. Each of the adult bird virus suspensions was injected into 40, 5-day-old (0.05 ml. inoculum i.c.) and 2, 485-day-old RIR chickens (inoculum in group 1 was 0.6 ml. i.p.; group 2 was 0.7 ml. i.p.). Results were as follows: group 1—16 out of 39 chicks were AE-positive, 2 out of 2 adults were positive; group 2—7 out of 31 chicks were AE-positive, 1 out of 2 adults was positive. These positive clinical diagnoses were confirmed histologically. Thus, it can be seen that AE virus resulting in ataxia in adult birds can be reisolated from the brain of the adult bird. When the brain suspensions were injected into susceptible chicks, the typical AE syndrome resulted. It was also of interest to note that the virus injected i.p. into adult birds apparently traveled to the brain, and also produced visceral lesions (table 2) which were not seen generally in i.c.-injected chicks.

Immunological Aspects

Neutralization Tests. — Neutralization tests in chicks (i.c.) showed that an appreciable antibody level had developed in chicks convalescent from experimental AE. Two groups of convalescent birds were produced and bled twenty-eight and forty-four days, respectively, after the first appearance of clinical symptoms. Serums from the first group showed at least 100 neutralizing doses (n.d.) to AE virus, while the second group showed at least 1,000 n.d. When the latter group was bled 226 days after the onset of symptoms, the serums showed 1,250 n.d. Normal or control serums showed 5 n.d. These results correlated with those obtained by Olitsky[11] and Jungherr and Minard.[7]

Hemagglutination Tests.—Following the procedure described by Olitsky and Yager[13] for certain encephalomyelitis viruses, hemagglutination in the cold could not be specifically produced using chicken, turkey, ox, and sheep red blood cells.

Susceptibility Variations.—A difference in susceptibility to AE virus was noted while conducting some of the initial experiments in chicks. In comparing a single strain source of RIR and SLX chicks for susceptibility to AE by i.c. challenge with 0.05 ml. of a 10^{-1} dilution of virus, the following picture was obtained. Seven days postinoculation, 11 of 20 RIR chicks appeared clinically positive to AE, while the SLX chicks were still negative. On the eighth day postinoculation, 2 SLX chicks appeared clinically positive. Ten days postinoculation, all of the RIR were clinically positive to AE, while only 7 out of 16 of the SLX chicks were positive. Twenty-eight days postinoculation, 15 of 16 of the SLX chicks were clinically positive to AE.

In coöperation with the University Poultry Department, experiments were undertaken to determine the difference, if any,

TABLE 2—Adult Bird Injection with Avian Encephalomyelitis Virus

			Microscopic pathology														
Bird No.	Amount (cc.) inoculum	Symptoms	Cerebellar molecular layer	Cerebellar nuclei	Cerebellar white matter	Brain stem	Forebrain	Choroid plexi	Ventriculus	Proventriculus	Duodenum	Pancreas	Spleen	Liver	Kidney	Heart	Oviduct
1	0.10	N35	–	–	VC	VC	–	–	LF	–	–	LF	–	LF	LF	LF	–
2	0.10	N35	G	VC	–	VC	–	–	–	–	–	–	–	LF	–	–	–
						VC											
3	0.25	A22	G	VC	–	G	–	–	–	–	–	CF	–	–	–	VC	–
4	0.25	A20	G	VC	–	VC	–	–	LF	LF	LF	–	–	–	LF	LF	–
				VC		VC											
5	0.50	A20	G	G	–	G	–	–	VC	–	–	–	–	–	LF	–	–
6	0.50	N43	–	–	–	–	–	–	–	–	–	CF	–	–	–	–	–
				VC		VC											
7	1.00	A20	G	G	–	G	VC	–	LF	–	LF	–	–	–	LF	LF	–
8	1.00	D13	VC	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	1.50	N43	–	VC	–	–	VC	–	–	–	–	CF	–	–	–	–	–
10	1.50	A14	VC	VC	–	–	–	–	–	–	–	–	–	LF	–	LF	–

A = ataxia, number indicates days postinoculation; sacrificed; N = clinically normal, number indicates days postinoculation; sacrificed; D = dead, number indicates days postinoculation; G = gliosis; VC = vascular cuffs; LF = lymphocytic foci; CF = circumscribed follicles; – = normal.

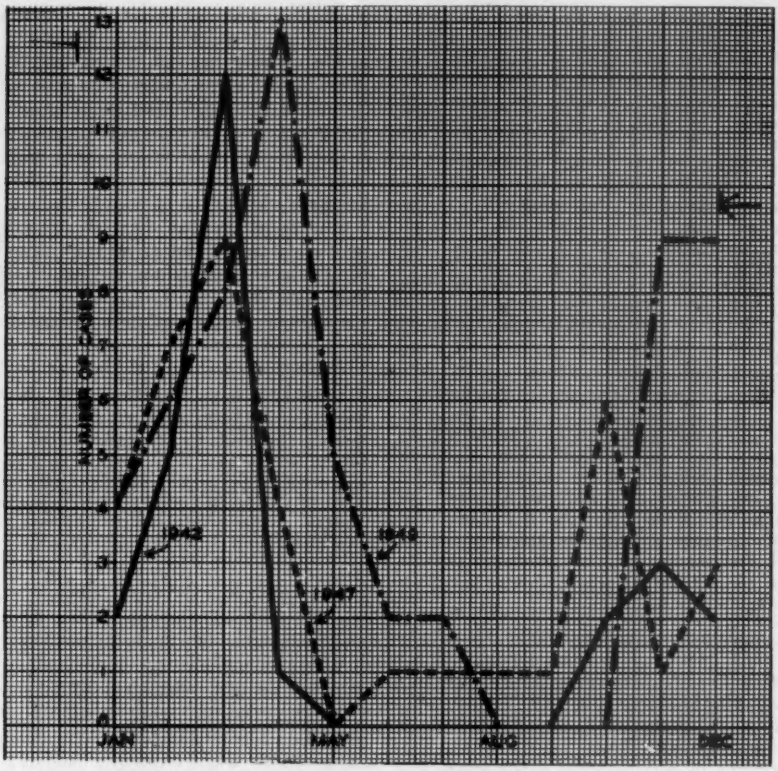

Fig. 1—Monthly incidence of avian encephalomyelitis in Connecticut.

in susceptibility of the following five strains of breeds to AE virus: RIR, SLX (NH male x BR female), BR, NH, and WR. The project was set up in two parts, each part conducted in duplicate. All chicks were vaccinated for Newcastle disease and infectious bronchitis, as previously described, at 1 day of age, brooded together until the twentieth day when they were injected i.c. with 0.1 ml. of an AE virus suspension, then randomly distributed throughout a brooder-battery. In part 1, a $10^{-4.5}$ dilution of the virus was used, this being determined as the dilution which should produce between 30 and 70 per cent infection in the RIR breed (previous virus titrations used as a basis). In part 2 (which was undertaken after the completion of part 1), a 10^{-3} dilution of the virus was used to bring the more resistant breeds (as determined in part 1) into the 30 to 70 per cent reaction group for a more accurate analysis of results. Control chicks (5 per breed) received a $10^{-4.5}$ or 10^{-3} dilution of a normal brain suspension. Chicks were called positive on the basis of clinical symptoms; at least 10 per cent of the positive chicks of each breed were examined histologically for confirmation of AE. Sex was determined on all chicks. Results are shown in table 3.

For the statistical analysis, experimental results in proper fractions were transformed to degrees[4] for more accuracy at the extremes, then retransformed to per cent. The difference due to strains of breeds was shown to be highly significant (part 1—$P>.001$; part 2—$P>.001$). No significant differences were found due to periods (duplicates), sex, or sexes within a breed. The incubation period of the virus in the SLX and BR chicks was shown to be longer than for the other breeds which reacted fairly uniformly. Thus, it can be seen that the RIR, NH, and WR were the most sus-

ceptible strains; BR were intermediate; and SLX were most resistant.

Pooled serum samples from chicks in each group were analyzed by means of the neutralization test in chicks. The serum samples were stored at —20 C. and heat-inactivated at 56 C. for thirty minutes before using. Equal parts of undiluted serum and virus dilutions were mixed and 10 chicks per dilution injected with 0.1 ml. of the mixture. No significant difference was noted between the serum samples from any of the five strains of breeds used. This indicated that the previously noted difference in strain susceptibility was apparently not due to neutralizing antibodies present in the blood of the chicks.

SUMMARY

1) From survey data of the geographical incidence of avian encephalomyelitis (AE), it is apparent that the disease has spread generally throughout the country since the last published report in 1942.

2) Data are given concerning field outbreaks of AE in Connecticut for the ten-year period, 1941 through 1950.

3) Avian encephalomyelitis continues to show a seasonal incidence with a minor period of prevalence in the fall and a major one in the spring months.

4) A "bead-mashing" technique of virus preparation is described which offers: (1) uniformity of resultant suspensions, (2) preparation at cold temperatures, (3) reduced chance of contamination, and (4) a potential for large pool-size preparations.

5) Day-old vaccination for Newcastle disease and infectious bronchitis eliminated interfering outbreaks of these diseases.

6) Penicillin, streptomycin, and aureomycin at described levels exhibited no inhibitory effect on AE virus.

7) Injection of $10^{4.4}$ minimal infective doses of AE virus did not produce lesions on the chorioallantoic membrane nor did it result in infected chicks hatched after an intra-amniotic injection of embryos.

8) A pleuropneumonia-like organism was isolated in serial yolk sac passages of AE virus. The organism evidently caused embryo mortality. Since this organism could not be isolated from the original AE virus inoculum, it was suspected that the organism was present in the eggs before inoculation.

9) Avian encephalomyelitis virus could be demonstrated in the yolk sac of embryonating eggs five and six days postinoculation (longest periods tested).

10) Duplicate zig-zag chicken embryo passages did not alter AE virus sufficiently for adaptation or multiplication in chicken embryos.

11) Contrary to reports by previous workers, AE virus could be demonstrated in the allantoic fluid of embryonating eggs as follows: embryos dead four, five, six, and seven days postinoculation; living embryos six and seven days postinoculation.

12) A death pattern could not be produced by five or six "blind" serial passages in the allantois of embryonating eggs.

13) The intraocular route was shown to be an effective method of transmitting AE to susceptible chicks, although not as effective as the intracerebral route.

14) Definite iridocyclitis was produced in about 50 per cent of the chicks injected intraocularly with AE virus.

15) Adult birds (195 to 444 days old) were successfully infected by intraperitoneal injection of AE virus. Brain and visceral lesions were seen on histological examination, similar to those found in chicks. The virus could be passed back to chicks intracerebrally.

16) Neutralization tests in chicks showed that an appreciable antibody level was developed in birds convalescent to AE.

TABLE 3—Breed Susceptibility to Avian Encephalomyelitis

	$10^{-4.5}$ experiment (part 1)						10^{-3} experiment (part 2)					
	Period 1		Period 2			*1	Period 1		Period 2			
Breed	Female	Male	Female	Male	Total affected	Ave. per cent affected	Female	Male	Female	Male	Total affected	Ave. per cent affected
RIR	8/20*	2/ 6	5/11	4/ 9	19/46	41	14/14	9/ 9	18/18	20/20	61/61	100
SLX	0/11	1/15	0/ 7	0/10	1/43	<1	5/ 9	6/14	9/16	10/21	30/60	50
BR	1/14	0/10	0/ 9	0/ 7	1/40	<1	7/11	10/13	11/14	13/22	41/60	70
NH	4/10	6/16	4/10	3/10	17/46	37	12/12	12/12	15/15	21/22	60/61	99
WR	5/10	9/15	5/10	3/ 9	22/44	48	16/17	8/ 8	19/19	19/19	62/63	99

* = affected/total. *1 = from statistical analysis—using angular transformation table showing transformation of degrees to percentages.

17) A hemagglutination test in the cold with AE virus proved ineffective using chicken, turkey, ox, and sheep red blood cells.

18) A statistically significant difference in susceptibility to AE was uncovered separating the available strains of breeds into the following groups: (1) most susceptible, RIR, NH, and WR; (2) intermediate, BR; (3) least susceptible, SLX. No significant difference was noted between serum samples from any of the five strains of breeds used when examined for neutralizing antibodies.

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Chicken Embryo Inoculation Procedures for Virus Cultivation

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REFERENCE TO all the studies in which chicken embryos have been used for virus cultivation would of necessity be most voluminous. We have observed that, except for minor variations, only a few basic procedures have been used. Beveridge and Burnet[1] gave a good review of the viruses which have been grown in eggs and mentioned most of the techniques used. Attention is only rarely directed, however, to the earliest studies of virus culture in eggs. We have attempted to review those which were published before Goodpasture's reports, and then to present only those features of other reports which offered new or improved techniques.

REVIEW OF EARLY LITERATURE

Centanni,[2] in 1902, was apparently the first to report attempts to culture a filtrable virus in embryonating hen eggs. He had observed that eggs laid during an epizoötic, as well as those taken from birds dying of fowl plague, failed to infect a healthy chicken fed for three days on a mixture of such eggs and bran. Chicks hatched from either healthy or infected breeders died readily during an epizoötic. This made it seem unlikely that virus was naturally present in the egg. He therefore infected each of four eggs with 2 drops of blood and sealed the hole with paraffin and collodion. The eggs were opened on the twenty-second day of incubation. In one, an embryo of 25 mm. was present; in another, 9 mm.; and in a third, only a firm mass of 5 mm. in an otherwise decomposed egg. The remaining egg showed no trace of embryo or decomposition. When the three nondecomposed eggs were given to a healthy hen, distinct symptoms of the disease appeared in five and one-half days.

Rous and Murphy,[3] in 1911, made a small window in the shell of an egg, and then tore a smaller opening in the shell membrane. Through this opening, minute portions of fowl sarcomas or sarcoma filtrates were injected "into the chick now plainly visible." The window was closed with the shell and sealed with pieces of moist shell membrane. They used embryos 6 to 16 days of age, and injections were made into the embryo or into an extra-embryonic cavity.

Jouan and Staub,[4] in 1920, described a procedure which they used to propagate fowl plague virus through six generations in eggs. A fine-pointed pipette charged with a trace of virus was thrust through a portion of the shell which had been heated in such a way that the albumen was not coagulated. Only the yolk was penetrated. The small hole was then sealed with Golaz's wax. Controls, inoculated only with physiological saline, developed normally.

Gay and Thompson,[5] in 1929, having recognized that cultures of embryonic chicken tissues appeared to be the most suitable for the growth of vaccine virus, attempted virus propagation in incubating hens' eggs. Inoculations were made in the yolk (details not given) after five to six days of preliminary incubation and, after inoculation, incubation was continued for four to ten days. In nearly every sterile, although usually dead embryo, living virus was found when tested intradermally in rabbits. A second series of eggs inoculated with yolk from the first series contained virus, but a third transfer was negative. They found that embryo tissue of the first passage contained more virus per unit volume than yolk, and concluded that series propagation might have been more successful had embryo tissue been used instead of yolk.

Chorioallantoic Membrane Inoculation.—In spite of the above reports, virus culture in embryonating eggs was not widely practiced until after the reports of Woodruff and Goodpasture,[6] in 1931, and Goodpasture, Woodruff, and Buddingh,[7] in 1931. Their original technique was that described by Clark,[8] in 1920. The egg was placed in a plasticene mold, with the pointed end up so that the air cell was bathed in water at 39 C. to prevent sagging of the egg contents when the shell overlying the embryo was opened. After disinfection of the operative surface with alcohol and a flame, a window 7 to 10 mm. was cut in the shell with a sharp point. After removal of the shell, the shell membrane was cut away to expose the chorioallantoic membrane (CAM). After the membrane was pricked with a needle, a drop of noncontaminated fowlpox virus was dropped on the injured area. The hole was closed by placing a ring of vaseline on the shell and then fixing on it a sterile cover slip. The egg was then incubated with the cover slip up-

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permost. Well-defined lesions appeared at four days, and very abundant lesions were present by seven days. Embryos 4 to 12 days of age were infected by this technique. Goodpasture *et al.*[7] described the growth of vaccinia and herpes simplex viruses in eggs and pointed out that, whereas these viruses grew poorly or not at all in adult chickens, good to excellent growth resulted in the embryo. Hence, they suggested that it would seem promising to test the infectiousness of materials from animal diseases that are known or suspected to be caused by a virus, but which have not been transmitted to an alien host. They added the suggestions that the shell be marked to indicate the position of the membrane and that this area be painted with paraffin to prevent pieces of shell from introducing infection when the egg is opened for inoculation.

Burnet,[9] in 1933, described a somewhat similar technique which he used in growing canary pox virus. He made some changes with a view toward forming an artificial air cell. A small drill hole was made in the shell and its membrane over the air cell. Through a triangular opening in the shell on the side of the egg above the embryo, a slit was made in the inner shell membrane. By virtue of its weight, the egg contents pulled away from the shell membrane around the triangular opening and filled the natural air cell. Inoculum was dropped through the opening into the artificial air cell onto the CAM. The opening into the natural air cell was then closed with a drop of paraffin, and the larger triangular hole was closed with a cover slip mounted on a rim of paraffin-vaseline mixture. Both Goodpasture,[10] in 1935, and Burnet, in 1942[11] and 1946,[1] later resorted to the use of a dental drill and abrasive disk to make the shell cuts. Burnet also advocated the use of a bulb or nipple over the air sac hole to aid in drawing down the egg contents to form the artificial air cell. A drop of broth placed on the inner shell membrane before the slit was made was reported to facilitate further the loosening of the CAM.

Brandly,[12] in 1934, at first introduced laryngotracheitis virus of fowls into the yolk of fertile eggs after about seven days' incubation, apparently in an effort to learn whether the virus might be transmitted through the egg. He followed the technique of Bouges,[13] described in 1930, which was simply to remove a small chip of shell near the pointed end of the egg and then, with egg held point down at an angle of about 45 degrees, to introduce the syringe needle into the lower part of the yolk sac. The hole was covered with plaster of paris. Later, in 1935 and 1936, Brandly described a different technique.[14, 15] An abrasive dental disk was used to cut the shell over the air cell along a circular line drawn on the shell about 2 mm. from the limits of the air sac. The small cap of shell, about 10 to 12 mm. in diameter, was lifted off with a sterile, sharp instrument. The egg was placed in a holder with the large end up. The bevel of a 22-gauge needle was turned down, and the needle was maintained almost parallel to the inner shell membrane while passing through it. Immediately after the needle had passed beneath the inner shell membrane, its point was slightly elevated to raise the membrane. Inoculum (0.01 to 0.2 ml.) was slowly injected. The egg was sealed by strips of Scotch tape, or by mounting a coverglass on a paraffin rim placed around the hole.

Alexander,[16] in 1938, described a slight modification of the procedure, in which an artificial air cell was formed. He simply bored a small hole over the air cell and then, by a back and forth motion of a dental drill, ground off the shell over a small area on the side of the egg over the embryo. The artificial air cell was formed, and inoculum introduced in the usual way. After this, because of the small size of the holes, it was only necessary to seal them with melted paraffin.

Beveridge and Burnet,[1] in 1946, described a modification of Alexander's technique. They placed a small drop of sterile liquid paraffin on the exposed shell membrane to clarify it and to make it nonwettable. The inoculum (0.05 ml.) was then placed on the shell membrane, and a puncture in the membrane was made with the end of a glass rod to which had been fused a sphere about 2/3 mm. in diameter. At the same time, gentle suction was applied with a rubber nipple on the hole over the air cell.

Allantoic Sac Inoculation.—Beveridge and Burnet,[1] in 1946, described the following technique for allantoic sac inoculation. A point was marked on the shell where the CAM was well developed but free of large vessels. A groove about 3 mm. long and 1 mm. wide was drilled through the shell without damaging the shell membrane. Inoculation was done with a syringe and needle by piercing the membranes for a few millimeters, or a Pasteur pipette was used to place a small drop of inoculum on the shell membrane. A dissecting needle was thrust through the drop and the membrane. As the inoculum flowed in, the remainder was deposited and it too flowed in. The hole was sealed with melted paraffin.

Hirst,[17] in 1942, briefly described a method of inoculation, but it is not clear whether the inoculum was deposited in the allantoic cavity or upon the CAM. A puncture was made over the CAM on the side of the egg, and another over the air cell. One-tenth milliliter of inoculum was injected into the hole over the CAM by placing the point of the needle "just within the shell." The holes were sealed with paraffin.

Yolk Sac Inoculation.—Both Cox,[18] in 1938, and Stimpert,[19] in 1939, recorded successful

yolk sac inoculations by simply boring a hole in the shell over the air sac. A 21-gauge needle was passed through the hole and down the center of the egg. Cox used needles 1¼ in. long. Paraffin was used to close the hole. Beveridge and Burnet[1] referred to the same technique.

Amnionic Sac Inoculation.—Burnet,[20, 21] in 1940 and 1941, and Beveridge and Burnet,[1] in 1946, described a method which consisted of forming an artificial air cell over the embryo. A cut about 5 cm. long was made in the overlying CAM, and the amnionic sac was lifted through the opening with curved forceps until taut. Injection of 0.05 to 0.25 ml. was made through the amnionic membrane. The opening was sealed with a coverglass and paraffin-vaseline mixture. Hirst,[25] in 1942, described a method which appears more often to have been an intra-embryonic injection. Holes were made in the blunt end of the egg and on the side over the embryo. A 1-in., 22-gauge needle was used to make a quick thrust in "the general direction" of the embryo. If the embryo were hit, it disappeared from view but could be made to reappear by moving the point of the needle nearer to the shell surface. This process, of course, was done over a strong light source. After inoculation, the holes were sealed with paraffin. Taylor and Chialvo,[23] in 1942, removed the shell cap over the air cell and dropped 50 per cent alcohol on the inner shell membrane to render it transparent. The closed points of fine curved forceps were thrust through the inner shell membrane and the CAM. Then the amnionic sac was lightly grasped and pulled up through the CAM. Injection was made into the base of the sac. The opened end of the egg was sealed with paraffin and a glass cap, and the egg was incubated in the upright position. Goodpasture and Buddingh,[24] in 1948, exposed the embryo by the window method, and then passed a needle through the CAM as close to the yolk sac as possible without penetrating it. The needle was pushed forward with the point directed toward the embryo. As this was done, the yolk was pulled toward the embryo by the thrust of the needle. The weight of the yolk served to pull the amnion back over the needle, and permitted the inoculum to be placed in the sac.

Experimental

Since 1935, a technique of inoculating eggs which has given good results has been employed in this laboratory. The air cells of fertile eggs are marked with a pencil. Usually 9- to 11-day-old embryos are used. The shell over the air cell is wiped with absorbent cotton dampened with 70 per cent ethyl alcohol and quickly flamed over a small alcohol flame. With a revolving carborundum disk mounted on the shaft of an electric motor, the shell is scored lightly in one plane by manually rotating the egg (fig. 1). In one place, the shell is cut down to, but not through, the outer shell membrane. With forceps that have been passed through a Bunsen flame, the cap of shell and the outer shell membrane are lifted off, exposing the inner shell membrane through a hole about 2 cm. in diameter (fig. 2).

For allantoic sac inoculations, the inoculum is deposited about ½ in. beneath the inner shell membrane with a 22-gauge needle. Chorioallantoic membrane inoculations are made by depositing the inoculum in a number of places on the CAM just beneath the inner shell membrane. For this purpose, an ordinary 22-gauge hypodermic needle is bent in a curve. With a little practice, one can rather consistently cause a bleb to form beneath the inner shell membrane, thus indicating that the inoculum has indeed gone on the CAM (fig. 2 and 3). When making yolk sac inoculations, it is sometimes helpful to clarify the inner shell membrane first by applying a thin oil. Although the yolk sac can be distinguished in most cases without the addition of oil, we find it rather difficult to penetrate with the needle. When the membrane has been rendered transparent, one can see that the needle point has actually penetrated the yolk sac before the inoculum is deposited.

When inoculation by any of the above methods has been completed, the egg is in-

Fig. 1—Scoring shell of eggs.

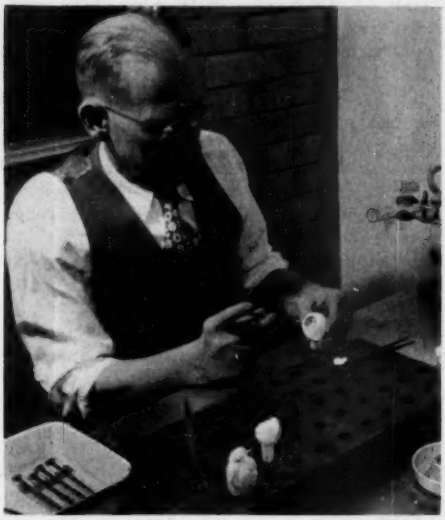

Fig. 2—Cap of shell removed from egg and inoculum being placed upon the chorioallantoic membrane.

verted on a piece of sterile bond letterhead paper. We use circular pieces, 9 cm. in diameter, packaged 50 to 55 to a Petri dish. They are autoclaved at 15 lb. steam pressure for forty-five minutes. Melted paraffin (Fisher tissuemat MP 60-62.5 C.) is then applied with a pipette and bulb so that the egg is sealed to the paper. After the paraffin has hardened, the egg and attached paper are removed from the Petri dish. Meanwhile, another egg has been inoculated so that it can be immediately placed on the newly exposed sterile surface, thus avoiding air contamination. The paper on the inoculated egg is trimmed with curved scissors to about a 0.5-cm. margin around the egg (fig. 4). Melted paraffin is then painted with a brush over the paper and the previously formed paraffin rim (fig. 5). One might suspect that paper could be cut originally to the size ultimately to be used. This we have found impractical, because melted paraffin flows off the edge of the top paper and sticks many papers together at their edges.

We have had difficulty in using forceps to elevate the amnionic sac without tearing it. Consequently, one of us (F.R.B.) suggested using a pipette for this purpose. The method ultimately used is as follows. At the time of marking air cells, eggs having strong embryos in the part of the egg nearest the air cell are selected for amnionic sac inoculation. The remaining eggs are used for other purposes. The opening over the air cell is made as previously described, but the egg is then immediately sealed to paper with paraffin. This provides a collar on which to stick the paper cover later. The egg is then placed with the small end in a supporting cup, and the paper cover is removed with sterile forceps and placed in a sterile Petri dish. A few drops of sterile oil are placed on the intact inner shell membrane, and an incision is made with a lance scalpel through the inner shell membrane and the CAM where there are no blood vessels, but over the embryo. By use of an apparatus described later, a shortened sterile serological pipette with attached nipple is lowered through the incision to the amnionic sac. The nipple, which is collapsed just before the pipette is inserted, is

Fig. 4—Trimming paper cap after sealing to egg with paraffin.

Fig. 3—Detail of chorioallantoic membrane inoculation.

released, thus sucking a portion of the amnion into it. When the pipette is retracted, the amnion will be drawn up and through the CAM incision so that injection with a hypodermic syringe and needle can be made within the amnionic sac (fig. 6). After inoculation, the bulb is again collapsed and the amnion discharged. The sterile paper cover is then taken from the Petri dish and replaced over the air cell opening. A gently heated spatula is used to seal it in place. After adhesion, the whole cap of the egg is painted with melted paraffin. The eggs are then returned to the incubator and incubated in an upright position.

The apparatus used for holding the serological pipette is a 10-cm. length of glass tubing (test tube) 18 mm. in diameter fitted with a No. 2 rubber stopper in each end. In the center of one of the stoppers, a hole is made to receive a 7-cm. length of glass tubing of about 7 mm. inside diameter. This glass tubing provides a channel through which the serological pipette, which is about 15 cm. long, slides. In the other stopper, a hole is made to receive a 1- by 8-cm. test tube, which is used to cover the pipette during and after sterilization. After assembly, the whole unit is wrapped in paper and sterilized in the autoclave at 15 lb. In operation, the large glass tube is supported by a burette clamp on a ring stand at a height and angle that will permit the pipette to be lowered sufficiently to reach the amnion, and raised sufficiently so that, when not in use, the tip of the pipette will be shielded within the large test tube. A rubber band from the ring stand to the pipette enables the operator to maintain the pipette at any desired level.

Fig. 5—Painting paraffin on the egg.

Fig. 6—Detail of amnionic sac inoculation.

With any of these routes of inoculation, harvesting is done by wiping the entire egg briskly with a piece of absorbent cotton held in each hand, the cotton having been soaked in 70 per cent ethyl alcohol. After the air cell end of the egg is flamed long enough to soften and slightly melt the paraffin, the paper cap is removed with sterile forceps, the shell and paraffin rim about the air cell are broken away, and the inner shell membrane is stripped off. The exposed CAM can then be inspected for lesions and, if desired, fluid can be collected directly from the egg. If preferred, the embryo and its membranes may be poured out in a Petri dish from which any portion may be used.

CONCLUSIONS

This general method of preparing eggs for inoculation has a number of advantages:

Adaptability.—By this technique, one can place his inoculum in any part of the embryo he desires. In addition to the methods described, a few embryos, on occasion, have been inoculated by the intravenous, intracephalic or intra-embryonic routes. We have also stripped off the inner shell membrane at the time of inoculation and suc-

cessfully transplanted bits of tracheal mucosa from adult birds.

Speed.—It is, in our experience, much easier to make one continuous circular cut around the air cell than several triangular cuts. At the same time, there is less danger to the embryo.

Visibility.—One is able to see in what part of the egg he is placing inoculum, particularly if a light mineral oil is dropped on the chorioallantoic membrane.

Harvest.—No further manipulation is needed between the time of inoculation and actual harvesting operation. In addition, when the paraffin rim is flamed, the melted paraffin spreads slightly down the side of the egg, thus decreasing the possibility of contamination.

Care of Eggs.—Since the egg has been subjected to a minimum of manipulation, it need not be incubated in the upright position. Candling is done as for uninoculated eggs. However, for eggs on which transplants of tissue have been made, or for amnionically inoculated eggs, we do incubate in the upright position.

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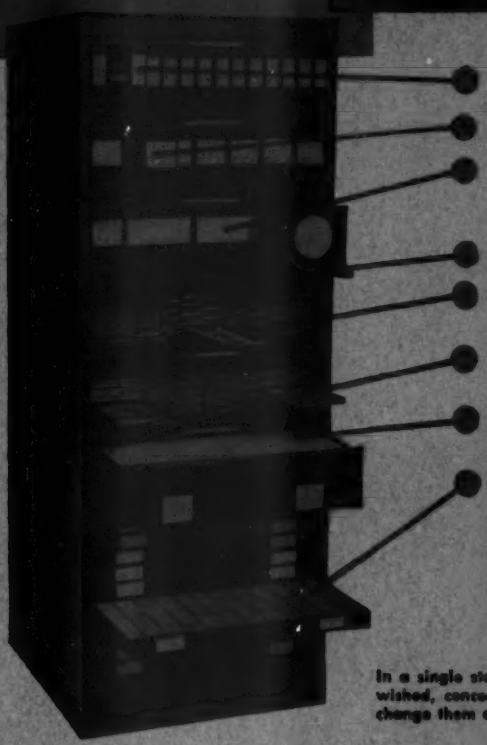

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